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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 31/415, 38/16, 31/20, 39/395, 31/05</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/18956</b> <b>(43) International Publication Date:</b> 22 April 1999 (22.04.99)
<b>(21) International Application Number:</b> PCT/US98/21570 <b>(22) International Filing Date:</b> 14 October 1998 (14.10.98)  <b>(30) Priority Data:</b> 60/062,335 15 October 1997 (15.10.97) US  <b>(71) Applicant:</b> CITY OF HOPE [US/US]; 1500 East Duarte Road, Duarte, CA 91010-0269 (US).  <b>(72) Inventors:</b> NATARAJAN, Rama, Devi; 16837 East Dawn Haven Road, Hacienda Heights, CA 91745 (US). NADLER, Jerry, L.; 2445 Upper Terrace Road, La Crescenta, CA 91214 (US).  <b>(74) Agents:</b> FIGG, E., Anthony et al.; Rothwell, Figg, Ernst & Kurz, Suite 701 East, Columbia Square, 555 13th Street N.W., Washington, DC 20004 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> 12(S)-HETE RECEPTOR BLOCKERS		
<b>(57) Abstract</b>  The 12-lipoxygenase product, 12(S)-HETE, mediates hyperproliferative and hyperplastic responses seen in atherosclerosis, diabetes, Parkinson's disease, Alzheimer's, stroke-induced nerve damage and cancer. 12-HETE also mediates inflammation and cell death in some cell systems, particularly B-islet cells of the pancreas. The present invention involves amelioration of disease states mediated by 12(S)-HETE by blocking specific 12(S)-HETE receptors.		

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**12(S)-HETE RECEPTOR BLOCKERS**

This application claims priority from provisional application 60/062,335, filed October 15, 1997.

**STATEMENT REGARDING FEDERALLY SUPPORTED RESEARCH**

5        This invention was made with government support under Grant No. DK 39721 awarded by the National Institutes of Health (NIDDK). The government may have certain rights in the invention.

**BACKGROUND OF THE INVENTION**

10    **Field of the Invention**

      The present invention relates to blockade of the 12(S)-HETE cell surface receptor as a treatment for conditions of the body which result from stimulation or overstimulation of the receptor. 12(S)-HETE, a product  
15    of the 12-lipoxygenase pathway, mediates the hyperproliferative and inflammatory responses present in such diseases as atherosclerosis, psoriasis, diabetes, and cancer. 12(S)-HETE also mediates inflammatory responses and cell death in some cell  
20    types, particularly pancreatic islet beta cells and nerve cells. Blockade of the 12(S)-HETE receptor

ameliorates the symptoms and arrests the mitogenic cellular responses.

### **Background**

Lipoxygenases (LO) are metabolic enzymes which  
5 catalyze the stereospecific oxygenation of  
polyunsaturated fatty acids to hydroperoxy fatty acids  
(Hamberg et al., J. Biol. Chem. 242:5329-5335 (1967)).  
The physiological function of 12-LO, the mammalian  
enzyme which catalyzes the oxygenation of arachidonic  
10 acid to (S)-12-hydroperoxyeicosatetraenoic acid (12-  
HPETE) and (S)-12-hydroxyeicosatetraenoic acid (12(S)-  
HETE), is unclear.

12-LO exists as two isoforms which are the  
products of different genes, leukocyte-type 12-LO and  
15 platelet-type 12-LO, which share 65% homology at the  
amino acid level (Izumi et al., Proc. Natl. Acad. Sci.,  
USA 87:7477-7481 (1990); Funk et al., Proc. Natl. Acad.  
Sci. USA 87:5638-5642 (1990)). The products of the 12-  
LO pathway, such as 12(S)-HETE, have been shown to play  
20 important roles in diseases such as atherosclerosis,  
diabetes, and cancer.

12(S)-HETE has direct mitogenic and hypertrophic  
effects in vascular cells. It is also a potent

chemoattractant for vascular smooth muscle cells (VSMC) and can activate oncogenes such as c-fos and ras and key growth-related kinases such as mitogen-activated protein kinases (ERK, JNK, PAK, p38) and protein kinase C. New results also indicate that 12(S)-HETE can directly increase monocyte binding. Human aortic endothelial cells incubated with 12(S)-HETE for four hours prior to monocyte adhesion assays resulted in an average increase of 3-fold (range of 1.5-5 fold) in monocyte binding as compared to untreated cells. In addition, glucose-induced monocyte adhesion was abrogated by the inhibition of 12-LO using both phenidone, a non-specific LO inhibitor, and baicalein, a more specific 12-LO inhibitor. The adhesion caused by 12-LO products appears to be monocyte-specific.

The 12-LO pathway is activated in pancreatic islets by cytokines and may participate in islet cell destruction. In inflammatory diseases, this pathway plays crucial roles in transmitting distinctive signals within the cell. Using inhibitors of the 12-LO enzyme pathway, researchers have been able to prevent inflammation and cellular damage. Furthermore, VSMC cultured under high glucose (HG) conditions produce increased amounts of 12(S)-HETE (Natarajan et al.,

Proc. Natl. Acad. Sci. USA 90:4947-4951 (1993). Thus, this pathway may be key to the accelerated cardiovascular disease observed in diabetes.

The LO pathway also plays a role in the growth-promoting effects of angiotensin II (AII) and in the chemotactic effects of platelet-derived growth factor: the products of the 12-LO pathway, and 12(S)-HETE in particular, are associated with the hypertrophic, hyperplastic, and mitogenic effects induced by AII.

Wen et al., 271 Am. J. Physiol. (40 Cell Physiol.) C1212-C1220 (1996); (Natarajan et al., Hypertension 23:1142-1147 (1994)). The proliferative effects of AII are inhibited by baicalein, a LO inhibitor. The mitogenic effects of 12(S)-HETE are similar to those of AII and are abrogated by pertussis toxin, implicating a G-protein mechanism. The 12-LO enzyme pathway is known to generate proinflammatory mediators in a variety of cells (O.R. Etingin et al., J. Lipid Res. 31:299-305 (1990); V.A. Folcik and M.K. Cathcart J. Lipid Res. 34:69-79 (1993)). Human and rat pancreatic B-cells specifically express active leukocyte type 12-LO (V.P. Shannon et al., Am. J. Physiol. 263:E828-E836 (1992); D.S. Bleich et al., Endocrinol. 136:5736-5744 (1995)). Recent evidence implicates products of the 12-LO

pathway in nerve cell death associated with Parkinson's disease, Alzheimer's disease and other inflammatory nerve cell conditions (Neuron 19:453-463 (1997)).

Because 12(S)-HETE has several biological effects  
5 linked to cellular growth in vascular smooth muscle and cardiac fibroblasts (Natarajan et al., Hypertension 23:1142-1147 (1994); Wen et al., Am. J. Physiol. 271:C1212-C1220 (1996)), it is implicated in the etiology of cardiovascular disease. Further evidence  
10 that 12(S)-HETE is responsible for the cellular responses seen in cardiovascular disease in diabetic patients includes the fact that monocyte binding to cultured human aortic endothelial cells increases in chronic high glucose conditions, and that this is  
15 coincident with increased formation of LO products such as 12(S)-HETE. (Kim et al., Diabetes 43:1103-1107 (1994)). Furthermore, treatment of aortic endothelial cells with 12(S)-HETE increases monocyte binding, likely by stimulating JNK activity and inducing CS-1.  
20 12(S)-HETE can also stimulate vascular endothelial growth factor (VEGF) gene expression in vascular smooth muscle (Am. J. Physiol. 273: H2224-H2231 (1997)). VEGF has been linked to angiogenesis in diabetic

retinopathy, tumor growth and atherosclerotic vascular disease.

12(S)-HETE is also regarded as a mediator of inflammation and hyperproliferation of the skin

5 (Arenberger et al., Skin Pharmacol. 6:148-151 (1993); Gross et al., J. Invest. Dermatol. 94:446-451 (1990)) and is therefore implicated in skin diseases.

12(S)-HETE has been shown to enhance tumor cell adhesion to endothelial cells. (Honn et al., Cancer  
10 Metastasis Rev. 13:365-396 (1994)). 12(S)-HETE can directly increase p21 activated kinase (PAK). The effect appears to be through activation of small GTP binding proteins such as RAC and through activation of PI3K.

15 The precise mechanisms of 12(S)-HETE action are not clear, however recent studies have shown that the LO product, 12(S)-HETE, activates c-jun amino terminal kinase (JNK) (Wen et al., Circ. Res. 81:651-655  
(1997)). JNK is a small GTP-binding protein and a  
20 member of the MAP kinase family which is involved in cellular growth, inflammation, and apoptosis (Force et al., Circ. Res. 78:947-953 (1994)) and in cell cycle progression through G<sub>1</sub> (Olson et al., Science 269:1270-1272 (1995)). Evidence shows that JNK can serve as a



positive or negative modulator of cell growth in different cells. Olson et al., 269 Science 1270-1272 (1995); Yan et al., 372 Nature 798-800 (1994). 12(S)-HETE activation of JNK may also be the mediator of  
5 cytokine-induced pancreatic B-cell damage (Bleich et al., Biochem. Biophys. Res. Commun. 230:448-451 (1997)).

Newer evidence indicates that the growth factor and potent vasoconstrictor AII, linked to type-1  
10 receptor activation, can activate JNK and PAK (Wen et al., Circ. Res. 81:651-655 (1997); Schmitz et al., Circ. Res. 82:1272-1278 (1998)). Furthermore, AII can modulate serum deprivation-induced apoptosis by increasing JNK activity in vascular smooth muscle  
15 cells, Sueror et al., Circulation, Supp. 1, I-281 (1994), mediated by lipids derived from the 12-LO pathway, such as 12(S)-HETE. This indicates that 12-LO products participate in JNK activation at least in part through G<sub>1</sub>-protein signaling. The ability of pertussis  
20 toxin to block the activation of JNK by 12(S)-HETE also supports the theory that 12(S)-HETE is a mediator of AII-induced JNK activation through a G<sub>1</sub>-mediated pathway.

While several studies have demonstrated the potent biological effects of lipoxygenase products, the mechanisms of action of these effects are not known. Some reports have hinted at the presence of 12(S)-HETE  
5 receptors on transformed cells. Binding sites for 12(S)-HETE have been detected in carcinoma cells (Herbertsson and Hammarstrom, FEBS 298:249-252 (1992), on melanoma cells (Liu et al., Proc. Natl. Acad. Sci. USA 92:9323-9327 (1995), and in a human epidermal cell  
10 line (Gross et al., J. Invest. Dermatol. 94:446-451 (1990); Suss et al., Exptl. Cell Res. 191(2):204-208 (1990)).

The 12(S)-HETE receptors described in carcinoma cells are cytosolic receptors (Herbertsson and  
15 Hammarstrom, Biochem. Biophys. Acta 1244:191-197 (1995)), activation of which may mediate 12(S)-HETE induced mRNA production of genes coding for the integrin  $\alpha_{\text{IIB}}\beta_3$  (Chang et al., Biochem. Biophys. Res. Comm. 176:108-113 (1991)). The localization of this  
20 receptor is different from plasma cell membrane receptors coupled to a G-protein and acting through second messengers.

12(S)-HETE receptors on the cell surface of murine melanoma cells have been described. These receptors

stimulate the second messengers diacylglycerol and inositol phosphate, via a G-protein mechanism, resulting in protein kinase C<sub>2</sub> activation. (Liu et al., Proc. Natl. Acad. Sci. USA 92:9323-9327 (1995)). The binding  
5 of 12(S)-HETE to these receptors was blocked by 13(s)-hydroxyoctadecadienoic acid, a LO metabolite of linoleic acid, ablating the 12(S)-HETE increased adhesion of the cells to fibronectin. These authors suggest 12(S)-HETE may act in a "cytokine" fashion to  
10 regulate responses of adjacent tumor cells, endothelial cells, and platelets.

Receptors for 15-HETE have been identified in mast/basophil (PT-18) cells and were shown to possess properties of G-protein-coupled receptors (Vonakis and  
15 Vanderhoek, J. Biol. Chem. 267:23625-23631 (1992). Specific binding of 15-HETE to these receptors stimulated 5-LO, and while 12(S)-HETE was found to be an effective competitor of [<sup>3</sup>H]15-HETE binding to PT-18 cells, suggesting that 12(S)-HETE binds to the specific  
20 15-HETE receptor, the binding of 12(S)-HETE did not stimulate the lipxygenase. Very recent studies have indicated the activation of a cell surface G-protein-coupled 5-HETE receptor in neutrophils (Capadici et al., J. Clin. Invest. 102:165-175 (1998)).

The high affinity 12(S)-HETE-specific receptors in a human epidermal carcinoma cell line were induced by  $\gamma$ -IFN (Gross et al., J. Invest. Dermatol. 94:446-451 (1990)). Saturation binding of 12(S)-HETE to these  
5 receptors did not stimulate cell growth, therefore, the function of these receptors in the skin is entirely speculative, and not related to the AII-induced cellular effects mediated by cell surface 12(S)-HETE receptors in fibroblasts overexpressing the AII  
10 receptor and potentially in vascular smooth muscle cells. Two recent studies have indicated two additional agents which could reduce 12(S)-HETE binding (Kemeny and Ruzicka, Agents Actions 32:339-342 (1991); Kemeny et al., Arch. Dermatol. Res. 283:333-336  
15 (1991)).

Specific inhibitors of 12-LO have been described. Gorins et al., J. Med. Chem. 39:4871-4878 (1996). In that study, a series of substituted (carboxyalkyl)benzyl ethers were found to be selective  
20 inhibitors of leukocyte-type 12-LO. These inhibitors of 12-LO acted by serving as structural analogs for the enzyme. Gorins et al., J. Med. Chem. 39:4871-4878 (1996). The 5-LO inhibitor, 2-phenylmethyl-1-naphthol (DuP654), has also been shown to specifically inhibit

binding of 12(S)-HETE to receptors on the human epidermal cell line SCL-II. Arenberger et al., Skin Pharmacol 6:148-151 1993).

In vivo, inhibition of 12-LO has lowered blood pressure in several models of hypertensive animals, including rats (Stern et al., Am. J. Physiol. 257:H434-H443 (1989); Nozawa et al., Am. J. Physiol. 259:H1447-H1780 (1990)). In addition, blockage of 12-LO activity has alleviated the growth-factor induced effects of 12-HETE in vascular cells. This, along with the known increased expression of 12-LO observed in animal models of diabetes (Gu et al., Am. Diabet. Assoc. Meeting (1996); Natarajan et al., Intl. Aldosterone Meeting (1998)) and diabetes induced accelerated atherosclerosis (Gerrity et al., Circulation 1175 (1997)) strongly implicate 12-HETE and the 12-LO pathway in the etiology of these diseases. The harmful effects of 12-LO activation are ameliorated by blocking the production of 12(S)-HETE, providing the rationale for a method of treatment which focusses on preventing 12(S)-HETE binding to its receptor.

There is currently no inhibitor of 12(S)-HETE receptor binding in clinical use. Due to the existence of several isoforms of 12-LO, blockage of the 12-HETE

receptor is a more specific and direct way to correct a disease state in which there is increased production of 12(S)-HETE or the receptors are up-regulated. This invention therefore, could provide the basis for the development of interventions to reduce cardiovascular disease, diabetes, and cancer.

#### SUMMARY OF THE INVENTION

The present invention relates to a method of inhibiting the effects of the LO product 12(S)-HETE by blocking 12(S)-HETE receptors comprising the administration of an effective amount of a 12(S)-HETE receptor antagonist or an antibody directed against a cell surface 12(S)-HETE receptor. The blockade of 12(S)-HETE receptors provides a means for ameliorating the proliferative and mitogenic effects of glucose, PDGF or AII-induced 12(S)-HETE production, or direct effects of 12(S)-HETE inflammatory actions.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the binding of tritiated 12(S)-HETE and DuP654 to CHO-AT<sub>1a</sub> cells at increasing concentrations of unlabeled 12(S)-HETE.

Figure 2 shows a competitive binding curve of tritiated and unlabeled 12(S)-HETE with CHO-AT<sub>1a</sub> cells.

Figure 3 shows the effect of the 12(S)-HETE receptor antagonist, DuP654, on AII- and 12(S)-HETE-induced  
5 growth in CHO-AT<sub>1a</sub> cells.

Figure 4 shows the effect of three agents which bind to CHO-AT<sub>1a</sub> cells, DuP654 (a 12(s)HETE receptor antagonist), Losartan (a specific AII<sub>1a</sub> receptor antagonist) and pertussis toxin, relative to 12(S)-HETE  
10 effects. Inhibition of labeled 12(S)-HETE binding sites on AT<sub>1aE</sub> and At<sub>1a27</sub> (2 cloned overexpressing AT<sub>1a</sub>) cells are shown and pSV neo mock transfected cells.

Figure 5 shows the effect of Losartan on AII and 12(S)-HETE-induced mitogenesis in CHO-AT<sub>1a</sub> cells.

Figure 6 shows the mitogenic effects of AII and  
15 12(S)-HETE on Psv neo mock transfected cells, AT<sub>1a</sub> expressing cells and AT<sub>1b</sub> expressing cells.

Figure 7 shows the time course of PAK activation by 12(S)-HETE (10<sup>-7</sup> M) in CHO-AT<sub>1a</sub> cells.

Figure 8 shows the inhibitory effect of transient  
20 transfection of CHO-AT<sub>1a</sub> cells by a PBD plasmid on 12(S)-HETE-induced PAK activation.

Figure 9 is a representative autoradiogram of phosphorylated MBP bands demonstrating inhibition of

12(S)-HETE induced PAK activity by the PI 3-kinase inhibitor, LY294002.

#### DETAILED DESCRIPTION OF THE INVENTION

Angiotensin II (AII) has been shown to stimulate,  
5 through the AII AT<sub>1</sub> receptor, 12-LO activity in murine  
macrophages, Scheidegger *et al.*, J. Biol. Chem.  
272:21609-21615 (1997), and in smooth muscle cells,  
Natarajan *et al.*, Proc. Natl. Acad. Sci., USA 90:4947-  
4951 (1993); Kim *et al.*, Atherosclerol. Thromb. Vasc.  
10 Biol. 15:942-948 (1995). Stimulation of the 12-LO  
pathway in murine macrophages resulted in an increase  
of monocyte chemotaxis (Scheidegger *et al.*, (in press,  
1997)), presumably through modification of LDL. This  
activity links AII activation of 12-LO to  
15 atherosclerotic disease.

The potential mechanisms of AII-induced mitogenic  
effects in a Chinese hamster ovary fibroblast cell line  
overexpressing the rat vascular type 1a AII (AT<sub>1a</sub>)  
receptor have recently been examined. See Wen *et al.*,  
20 Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220  
(1996). AII had mitogenic effects in these cells,  
leading to a sustained increase in DNA synthesis as  
well as cell number. It was also observed in these



cells that the 12-lipoxygenase product, 12(S)-HETE, also had direct mitogenic effects in these cells. See Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996). Furthermore, 12(S)-HETE did not  
5 have any mitogenic effects in mock transfected cells. The addition of 12(S)-HETE to these CHO-AT<sub>1a</sub> cells led to a significant increase in the activity of the key growth-related kinases, mitogen activated protein kinase (Wen et al., Am. J. Physiol. 270 (Cell Physiol.  
10 40): C1212-C1220 (1996)), and c-jun amino terminal kinase (Wen et al., Circ. Res. 81:651-655 (1997)). This work has suggested that over expression of the AT<sub>1a</sub> receptor plays a role in inducing a putative 12(S)-HETE receptor, which is supported by the observation that  
15 the mitogenic effects of 12(S)-HETE were completely abrogated by pretreatment of the cells with pertussis toxin. Thus, the effects of 12(S)-HETE may be mediated by a Gi protein-coupled receptor. See example 3, below. Application of AII to CHO-AT<sub>1a</sub> cells resulted in  
20 a 2-fold increase in 12(S)-HETE formation and cell proliferation. These proliferative effects were inhibited by the 12-LO inhibitor, baicalein.

In accordance with the present invention, a 12-HETE receptor has been discovered and characterized.

For the first time, a specific high affinity 12(S)-HETE receptor has been identified. Chinese hamster ovary (CHO) fibroblasts that stably overexpress the rat vascular angiotensin type 1a receptor (CHO-AT<sub>1a</sub>) have been found to carry this receptor. This receptor is not present in mock transfected cells. Experiments have been performed which indicate that this receptor has characteristics of a G-protein coupled receptor. Furthermore, there is evidence of crosstalk between this receptor and the AT<sub>1b</sub> receptor, since a specific antagonist, Losartan, was able to partially block the binding of 12(S)-HETE to the cells and also blocked the mitogenic effects of 12(S)-HETE. Furthermore, a 12(S)-HETE receptor antagonist blocked 12(S)-HETE mitogenic effects and partially blocked AII mitogenic effects. Increased actions of vasoactive and growth promoting agents, such as angiotensin II, under pathologic conditions may up-regulate 12(S)-HETE receptors. Hence, further studies of this receptor in vascular and other cells, as well as the development of specific receptor antagonists, are expected to be therapeutically important.

It has also been found that hyperglycemic conditions result in both increased monocyte binding to

human aortic endothelial cells (HAEC) and increased 12(S)-HETE and 15-HETE activity. Neutrophil binding is not increased. In HAEC incubated in vitro with 12-LO products, increased monocyte binding, JNK activation, and induction of CS-1 fibronectin were detected, suggesting that the upregulation of 12-LO activity seen in hyperglycemia may exacerbate atherosclerosis by stimulating adhesion of monocytes through JNK activation and CS-1 production. For example, monocytes inabated with  $10^{-7}$  M 12(S)-HETE for 12 minutes at room temperature or at 37°C prior to monocyte adhesion assay demonstrated increased adhesion over untreated cells. See Example 10.

Blockade of 12(S)-HETE receptor binding therefore is a new method of treating disorders associated with increased 12-lipoxygenase expression and activity. These diseases include atherosclerotic cardiovascular disease, glucose and diabetes-induced complications, cytokine-induced inflammatory cellular effects, and tumor cell growth and metastasis.

The kinetics of radioactive [ $^3\text{H}$ ]12(S)-HETE binding to these cells at 4°C have been examined. These studies have revealed the presence of specific high affinity binding sites for 12(S)-HETE on these cells.

Specificity was determined by the observation that this binding of tritiated 12(S)-HETE was displaced by unlabeled 12(S)-HETE. A one site fit model yielded a  $K_d$  of 38.4 nM. See Example 1. The binding kinetics of [3H]12(S)-HETE have revealed the presence of specific high affinity 12(S)-HETE binding sites on CHO-AT<sub>1</sub> cells, but not in mock transfected cells; these results suggest that AII-induced mitogenic effects involve the production of reactive oxygen species and LO products via activation of G-protein-coupled receptors.

DuP654 could completely inhibit 12(S)-HETE-induced mitogenic effects. DuP654 significantly reduced cell growth induced by either AII or 12(S)-HETE at a concentration of 0.1  $\mu$ M. Tritiated 12(S)-HETE binding was also blocked by pertussis toxin (Figure 3). Pertussis toxin has been shown to ablate 12(S)-HETE-induced mitogenic effects (Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996)), implicating the involvement of a G<sub>i</sub> protein-coupled receptor. Losartan, a specific angiotensin AT<sub>1a</sub> receptor antagonist now in clinical use for the treatment of hypertension, partially blocked tritiated 12(S)-HETE binding (Figure 4). Similarly, it partially blocked 12(S)-HETE-induced mitogenic effects in these

CHO-AT<sub>1a</sub> cells, while fully inhibiting AII-induced proliferative effects (Figure 5). 12(S)-HETE had mitogenic effects only in CHO-AT<sub>1a</sub> cells, but not in mock transfected cells (pSVneo), nor in CHO cells overexpressing the angiotensin AT1b receptor (Figure 6).

This invention involves a method for inhibiting the effects of 12(S)-HETE by administration of an effective amount of a 12(S)-HETE receptor antagonist. The method is useful for the treatment or prophylaxis of conditions in which 12(S)-HETE receptor activation contributes to adverse effects. For example, the method of this invention may be employed for the treatment or prophylaxis of atherosclerotic cardiovascular disease, glucose-induced complications of diabetes, cytokine-induced inflammatory diseases and tumor cell growth and metastasis.

The 12(S)-HETE receptor antagonist may be any agent that blocks or significantly inhibits binding of 12(S)-HETE to its receptor. Such agents include DuP654 (2-phenylmethyl-1-naphthol), Losartan (2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole, potassium salt), pertussis toxin, 12(S)-HETE analogs, peptides and

peptide analogs having affinity for the binding site on the 12(S)-HETE receptor (especially antibodies which can block 12(S)-HETE receptors), antibodies to the 12(S)-HETE receptor, and the like.

5           The determination of appropriate, well-tolerated dosage forms for administration to humans for use in the present invention is within the ordinary skill in the art. Such dosage forms include tablets, capsules, syrups, suspensions, drops, injectable solutions,  
10   lozenges, implants, transdermal patches, and other dosage forms well known in the art for enteral or parenteral administration. Based on in vitro experiments on the effect of 12(S)-HETE blocking drugs on 12(S)-HETE binding, a dose of between about 0.5 and  
15   about 30 mg/kg/day would be effective in blocking 12(S)-HETE receptors in humans in vivo, and preferably from about 1 to about 10 mg/kg/day.

          The present invention is further illustrated by the following examples, which are not intended to be  
20   limiting.

EXAMPLESExample 1

Kinetics of [ $^3\text{H}$ ]12(S)-HETE binding to CHO-AT<sub>1a</sub> cells. Figures 1 and 2 are a competition curves which  
5 examine the specificity of [ $^3\text{H}$ ]12(S)-HETE binding. CHO-AT<sub>1a</sub> cells were grown to confluence in 24 well tissue culture dishes in HAM's F12 medium containing 10% fetal calf serum. The cells were then rinsed and placed in fresh medium, HAM's F12/HEPES with no other additives  
10 (450 ul per well). Serial dilutions of unlabeled 12(S)-HETE or DuP654 were added to the wells. Commercial unlabeled 12(S)-HETE (BioMol Corp.) was dried and reconstituted in ethanol to obtain a stock solution of 5 mM. DuP654 was dissolved in DMSO to get  
15 a stock solution of 5 mM. These were then serially diluted and added in a volume of 1 ul to the wells to obtain the final concentrations indicated. Then [ $^3\text{H}$ ]12(S)-HETE (10,000 cpm in a volume of 50 ul per well) was added from a stock solution obtained by  
20 adding the tracer to the medium. The plates were then incubated at 4°C with continuous shaking for 2 hr. The cells were then washed 2 times with cold PBS and lysed in 0.3N NaOH (200 ul). Radioactivity in the cell lysates was quantitated in scintillation counter.

Affinities and binding constants were obtained using Matlab computer software (Mlab, Civilized Software Inc., Bethesda, MD).

This experiment revealed the presence of specific high affinity binding sites for 12(S)-HETE on these cells. A one site fit model yielded a Kd of 38.4 nM. Specificity was determined by the observation that this binding of [<sup>3</sup>H]12(S)-HETE was displaced by unlabeled 12(S)-HETE.

10

#### Example 2

Reduction of cell growth induced by AII or 12(S)-HETE.

DuP654 significantly reduced cell growth induced by either AII or 12(S)-HETE at a concentration of 0.1  $\mu$ M. Complete inhibition of 12(S)-HETE induced mitogenic effects was seen. See Figure 3.

15

#### Example 3

Blockade of the 12(S)-HETE receptor by a specific antagonist.

20

Tritrated 12(S)-HETE binding is blocked by unlabeled 12(S)-HETE. See Figure 4. DuP654, a 12(S)-HETE receptor antagonist, was shown also to block 12(S)-HETE at a concentration of 0.1  $\mu$ M in both AT<sub>1a</sub>2 and AT<sub>1a</sub>27 cell types, two clones of CHO cells which



overexpress the  $ATI_{1a}$  receptor. See Figure 4. The cells were grown as described in Example 1.

#### Example 4

Blockade of 12(S)-HETE binding by pertussis toxin.

5 Cells were grown as described in Example 1. Prior to addition of drug (12(S)-HETE or DuP654) to the cells, the cultures were preincubated in HAM's F12 medium + 0.1% BSA for two hours at 37°C with or without 100 ng/ml pertussis toxin. Serial dilutions of unlabeled  
10 12(S)-HETE or DuP654 were added, followed by [ $^3H$ ]12(S)-HETE as described for Example 1. After incubation and washing, radioactivity in the cell lysates was quantitated. See Figure 4. As discussed above, pertussis toxin could also ablate 12(S)-HETE-induced  
15 mitogenic effects. This implicates the involvement of a  $G_i$ -protein-coupled receptor.

#### Example 5

Partial blockade of 12(S)-HETE mitogenesis by the specific angiotensin  $ATI_{1a}$  receptor antagonist, Losartan,  
20 in CHO- $ATI_{1a}$  cells. (Figure 5)

CHO- $ATI_{1a}$  cells were plated in 12-well dishes (about 5-10,000 cells per well) for 24 hr. in growth medium consisting of HAM's, F12 + 10% FCS. They were then serum depleted for 72 hours by replacing the medium

with HAM's F12 + 0.1% BSA. This medium was then freshly replaced along with AII or 12-HETE (0.1  $\mu$ M each) prior to addition of drug to the cells. Losartan was added as a solution in water to the cells 15 min. prior to the addition of AII or 12-HETE. The final concentration of Losartan was as indicated in Figure 4. Fresh medium containing the same concentrations of AII or 12(S)-HETE plus Losartan was replaced every 48 hours. At the end of 8 days, the medium was removed, and 1 ml trypsin was added per well followed by 1 ml isotone after 3 min. These trypsinized cells were counted on a Coulter counter. Losartan partially blocked 12(S)-HETE-induced mitogenic effects and fully blocked AII-induced proliferative effects. See Figure 5). Losartan also partially blocked [ $^3$ H] 12-HETE binding (Figure 4).

#### Example 6

Dependency of 12(S)-HETE mitogenic effects on expression of the AT<sub>1a</sub> receptor.

The three cell lines, CHO-AT<sub>1a</sub>, CHO-AT<sub>1b</sub> and mock transfected CHO cells were gifts from Dr. Eric Clauser (Inserm Unit, Paris, France). These cells were plated in 12 well dishes in HAM's F12 medium + 10% FCS. After 72 hours serum depletion in HAM's F12 + 0.05% FCS, the

cells were treated with AII or 12(S)-HETE (0.1  $\mu$ M). Cells counts (after trypsinization) were taken at 48 hour intervals and fresh medium along with AII or 12-HETE added at these 48 hour intervals. 12(S)-HETE had mitogenic effects only in CHO-AT<sub>1a</sub> cells, but not in mock transfected cells (pSVneo), nor in CHO cells overexpressing the angiotensin AT<sub>1b</sub> receptor. See Figure 6.

#### Example 7

PAK activation by 12(S)-HETE.

CHO-AT<sub>1a</sub> cells were gently washed and placed in depletion medium (HAM's F-12 medium containing 1 mg/ml BSA and 20 mM HEPES, pH 7.4) for 72 hours prior to use. After incubation for 30 minutes, the cells were treated with 10<sup>-7</sup>M 12(S)-HETE or with ethanol. The 12(S)-HETE treatment was terminated by washing twice with PBS and adding 300  $\mu$ l lysis buffer (50 mM HEPES, pH 7.5, containing 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1% NP-40, 2.5% glycerol and 1 mM Na<sub>3</sub>VO<sub>4</sub> containing the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and aprotonin) followed by sedimentation at 14,000 xg at 4 °C for ten minutes. Protein determination was performed by the Bradford method. The top panel shows

a representative autoradiogram of phosphonycated myelin basic protein (MBP) bands from a gel. PAK activity was measured as follows. First, 300 µg of lysate protein was incubated with PAK antibody (1:20) in lysis buffer overnight at 4°C, followed by incubation with 60 µl of a 50% slurry of protein A beads for 60 minutes. After washing three times with lysis buffer and twice with kinase buffer (50mM HEPES, pH 7.4, 10mM MgCl<sub>2</sub>, 10mM MnCl<sub>2</sub>, and 0.2 dithiothreitol) containing 2 µl MBP, 20 µM ATP and 5µCi [γ-<sup>32</sup>P] ATP, the kinase activity was measured in 60 µl kinase buffer. After incubation for 30 minutes at 30°C, the reaction was stopped with 5x Laemmli sample buffer and resolved on a 12% SDS-polyacrylamide gel, followed by autoradiography. The bottom panel shows the densitometric quantitation of PAK activity stimulated with 10<sup>-7</sup> M 12(S)-HETE or ethanol (control) for the time indicated. Each point is an average (mean ± SE) from at least 3 separate experiments. Results are expressed as stimulation over control.

#### Example 8

Inhibition of 12(S)-HETE induced PAK activation by transient transfection by a PAK binding domain (PDB) plasmid.

The degree of 12(S)-HETE induce PAK activation was compared in CHO-AT<sub>1a</sub> cells which had been transiently transfected with a PDB plasmid and cells which had not been transfected.

5 For the PBD-transfected group, CHO-AT<sub>1a</sub> cells were transiently transfected with 15 µg PBD plasmid. For the non-PBD-transfected group, CHO-AT<sub>1a</sub> cells were treated with the same transfection reagents as the PBD-transfected group, but lacking plasmid. Plasmids used  
10 were endotoxin-free and prepared by EndoFree plasmid kit (Qiagen Co.) with the standard protocol. The DNA transfection method used was a cationic liposome-mediated transfection with DOSPER transfection reagent (Boehringer Mannheim Co.) following the manufacturer's  
15 instructions. Briefly, the cells were plated the day before the transfection experiment at  $3 \times 10^6$  cells per 100 mm dish. The next day, cells were washed with Opti-MEM<sup>®</sup> reduced serum medium (Gibco BRL) and incubated in 5 ml of HAM's F-12 medium containing 1%  
20 FBS. Plasmid mixture (45 µg DUSPER/15 µg) was prepared and added to each dish. After a 5 hour incubation, the transfection medium was replaced and 8 ml fresh depletion medium (described in Example 7) continuing 1% FBS for overnight incubation was added. The cells were

washed twice with depletion medium, incubated in the same medium for another 32 hours and harvested. Cells were then treated with  $10^{-7}$ M 12(S)-HETE or ethanol for 10 minutes. The top panel of Figure 8 illustrates a representative autoradiogram of phosphorylated MBP bands from a gel. The bottom panel illustrates the densitometric quantification. Each point is an average (mean  $\pm$  SE) of at least 3 separate experiments. Results are expressed as stimulation over control. The PAK activity was measured as described in Example 7.

#### Example 9

Inhibition of PI 3-kinase by LY294002.

Cells were pretreated with different concentrations of the PI-3 kinase inhibitor, LY294002 or DMSO (control) for 30 minutes, then treated with  $10^{-7}$  M 12(S)-HETE or ethanol (control) for 10 minutes. PAK activity was measured as described in Example 7. Figure 9 shows a representative autoradiogram of phosphorylated MBP bands from 3 similar experiments.

20

#### Example 10

Increased monocyte adhesion to HAEC by treatment of monocytes with 12(S)-HETE.

Monocytes were incubated with  $10^{-9}$ M 12(S)-HETE for 12 minutes at room temperature (RT) or 37°C or left

untreated at room temperature and assayed for monocytes adhesion. Eight fields were counted for each experiment. Results are presented in Table 1.

Table 1 effect of 12(S)-HETE on Monocyte Adhesion to  
5 Human Aortic Endothelial Cells

Experiment	NT	12(S)-HETE (RT)	12(S)-HETE (37°C)
1	26.4 ± 12.5	39.6 ± 12.5 <sup>1</sup>	39.0 ± 9.3 <sup>3</sup>
2	28.3 ± 5.0	45.4 ± 7.6 <sup>2</sup>	ND <sup>4</sup>

- 10 1. P = 0.016  
2. P = 0.001  
3. P = 0.01  
4. ND = not done

**CLAIMS**

1. A method for inhibiting the effects of 12-HETE comprising administration of an effective amount of a 12(S)-HETE receptor blocker.

2. The method of claim 1 wherein the receptors are on the cell surface.

3. The method of claim 2 wherein the in the cells are selected from the group consisting of monocytes, endothelial cells, pancreatic islet beta cells, nerve cells, cardiac fibroblasts, cardiac  
5 myocytes and vascular smooth muscle cells.

4. The method of claim 1 wherein the administration of the compound is to an animal.

5. The method of claim 4 wherein the animal is a mammal.

6. The method of claim 4 wherein the animal is a human.

7. The method of claim 1 wherein the blocking of the receptor inhibits 12(S)-HETE binding.

8. The method of claim 1 wherein the blocking of the receptor inhibits receptor activation.

9. The method of claim 1 wherein the blocking of the receptor inhibits cell growth.



10. The method of claim 1 wherein the blocking of the receptor inhibits inflammatory cell damage.

11. The method of claim 1 wherein the blocking of the receptor inhibits cell death.

12. The method of claim 1 wherein the blocking of the receptor reduces monocyte adhesion.

13. The method of claim 1 wherein the blocking of the receptor reduces VEGF production.

14. The method of claim 1 wherein the blocking of the receptor reduces PAK activation.

15. The method of claim 1 wherein the 12(S)-HETE receptor blocker is a 12(S)-HETE receptor antagonist.

16. The method of claim 1 wherein the 12(S)-HETE receptor blocker is an antibody.

17. A method of suppressing the activation of 12(S)-HETE receptors comprising the administration of a compound which prevents the binding of endogenous receptor agonists to the receptor.

18. A method for the treatment or prophylaxis of a disease in which 12(S)-HETE receptor activation contributes to adverse effects which comprises administering an effective amount of a 12(S)-HETE  
5 receptor blocker.

19. The method of claim 18, wherein the disease is atherosclerotic cardiovascular disease, glucose-induced complications of diabetes, Parkinson's disease, Alzheimer's disease, stroke-induced nerve damage,  
5 cytokine induced inflammatory disease or tumor cell growth or metastasis.

20. The method of claim 19, wherein the 12-HETE receptor blocker is selected from the group consisting of DuP654, Losartan, pertussis toxin, a 12(S)-HETE analog, an antibody to the 12(S)-HETE receptor, a  
5 peptide which binds to the 12(S)-HETE receptor and a peptide analog which binds to the 12(S)-HETE receptor.

21. The method of claim 18, wherein the 12-HETE receptor blocker is administered in a dose of from about 0.5 to about 300 mg/kg/day.

22. The method of claim 21, wherein the 12-HETE receptor blocker is administered in a dose of from about 1 to about 10 mg/kg/day.

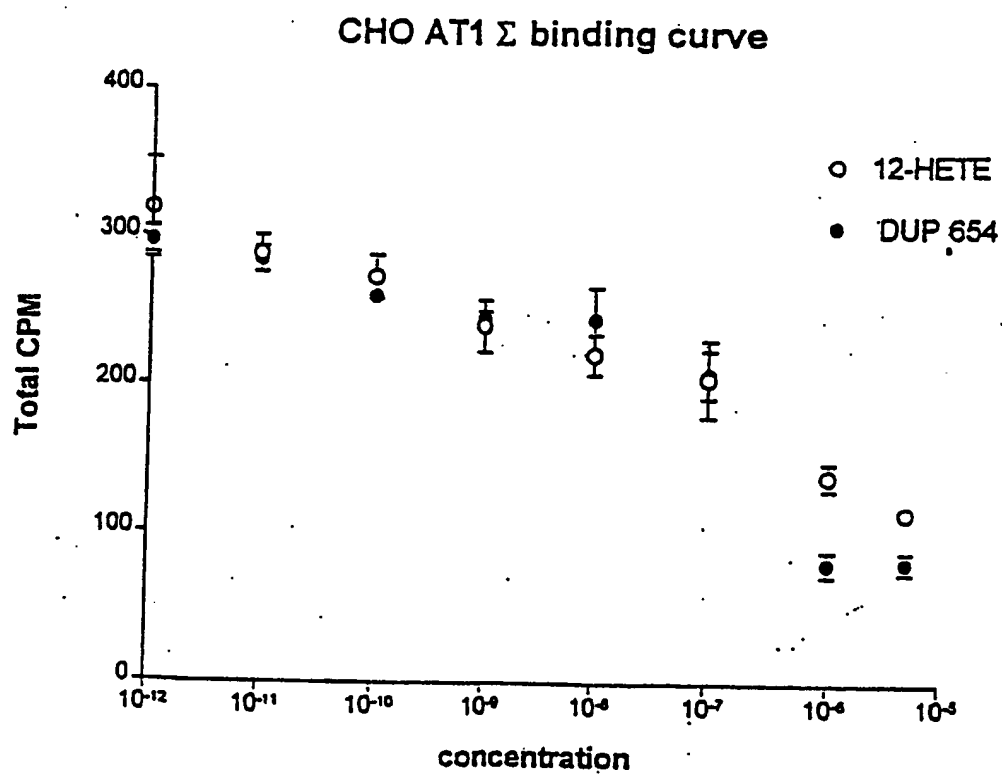
**FIGURE 1****[<sup>3</sup>H]12(S)-HETE Binding to CHO-AT<sub>1a</sub> Cells**

FIGURE 2

# Competition of Cold 12(S)-HETE for 12(S)-[<sup>3</sup>H]HETE Binding to CHO-AT<sub>1a</sub> Cells.

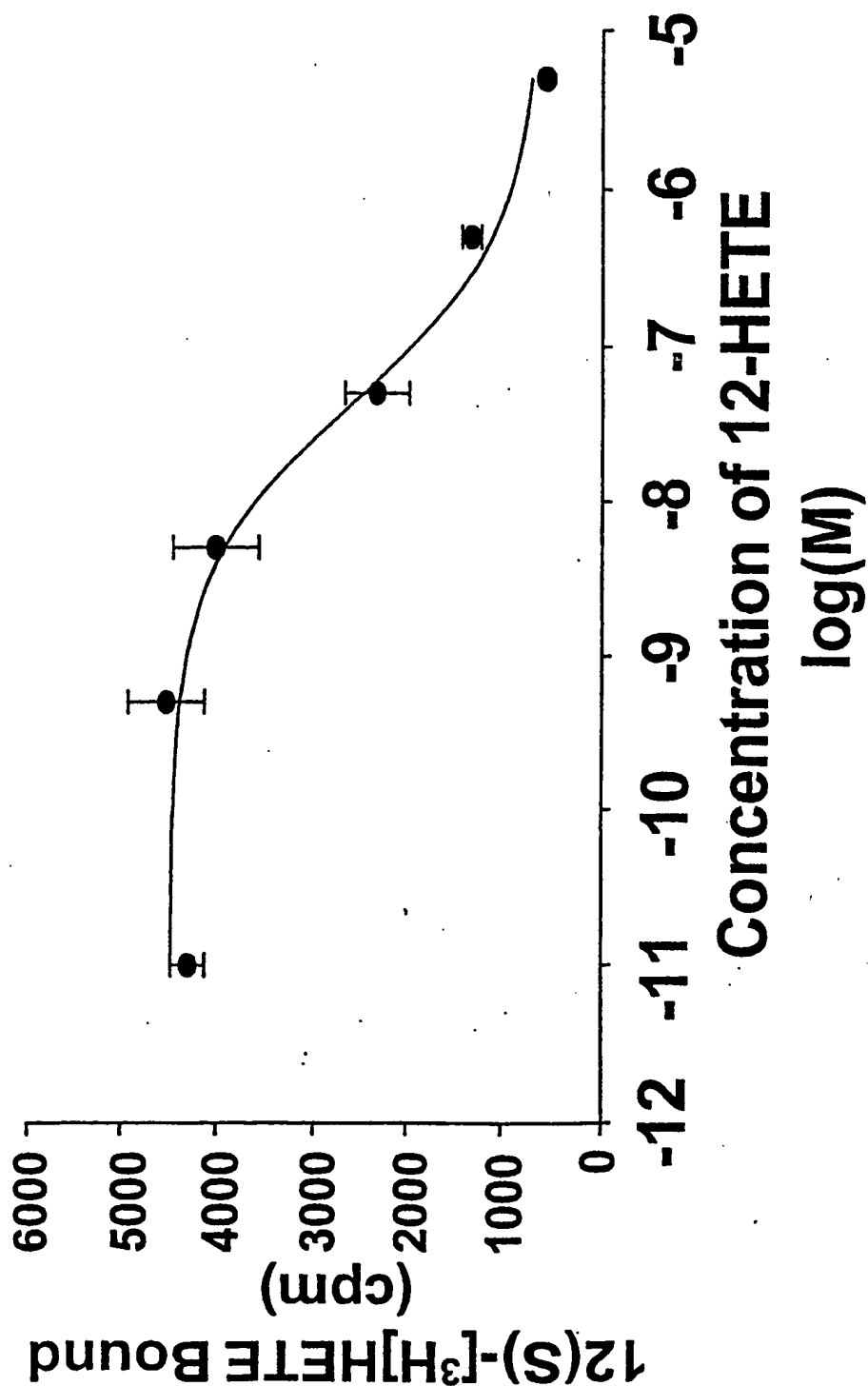


FIGURE 3

# Effect of DuP654(12-HETE receptor antagonist) on All and 12-HETE- induced Growth in CHO-AT<sub>1a</sub> cells

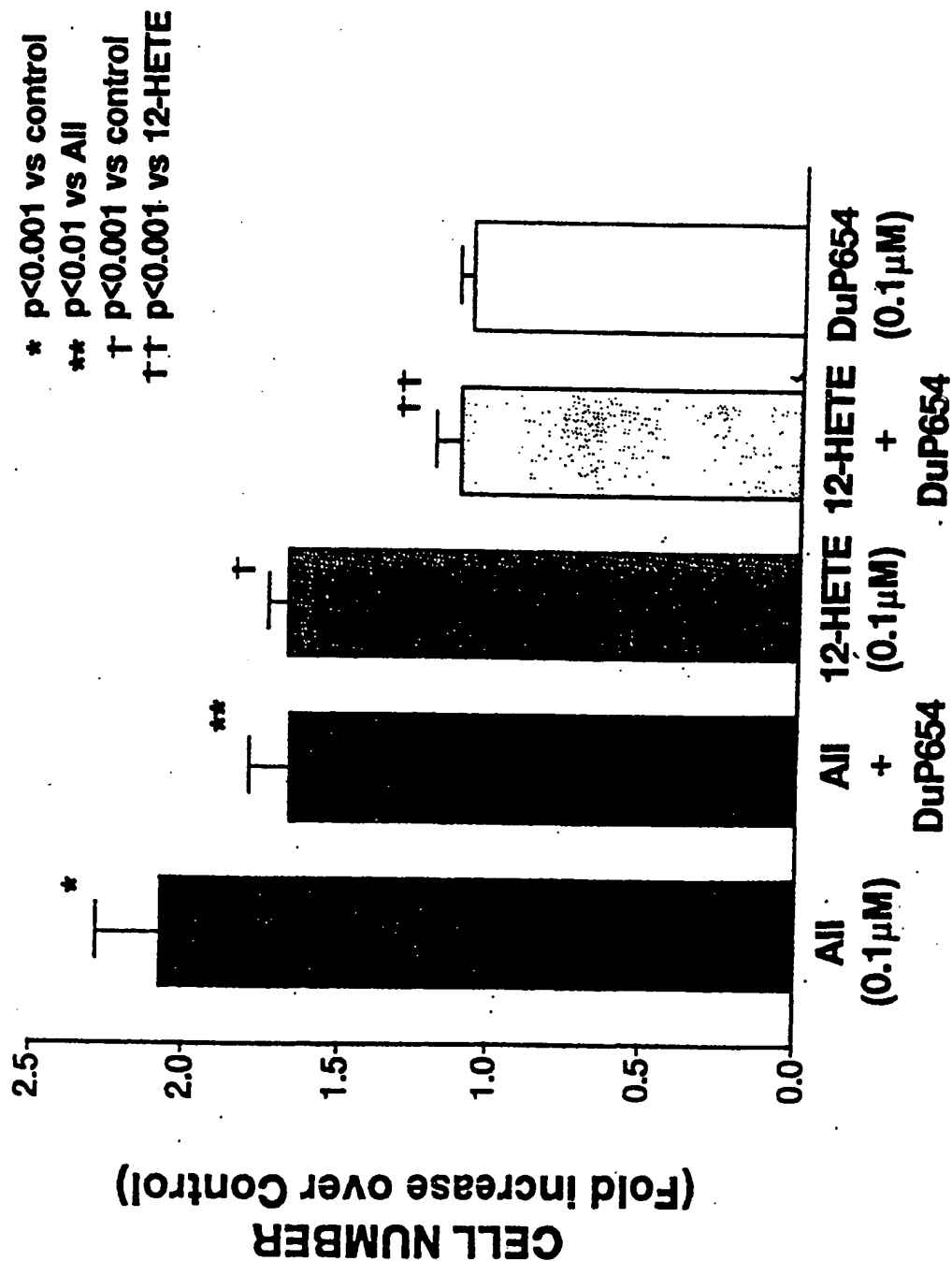


FIGURE 4

# Effect of Agents on 12(S)-[<sup>3</sup>H] HETE Binding to CHO-AT<sub>1a</sub> Cells

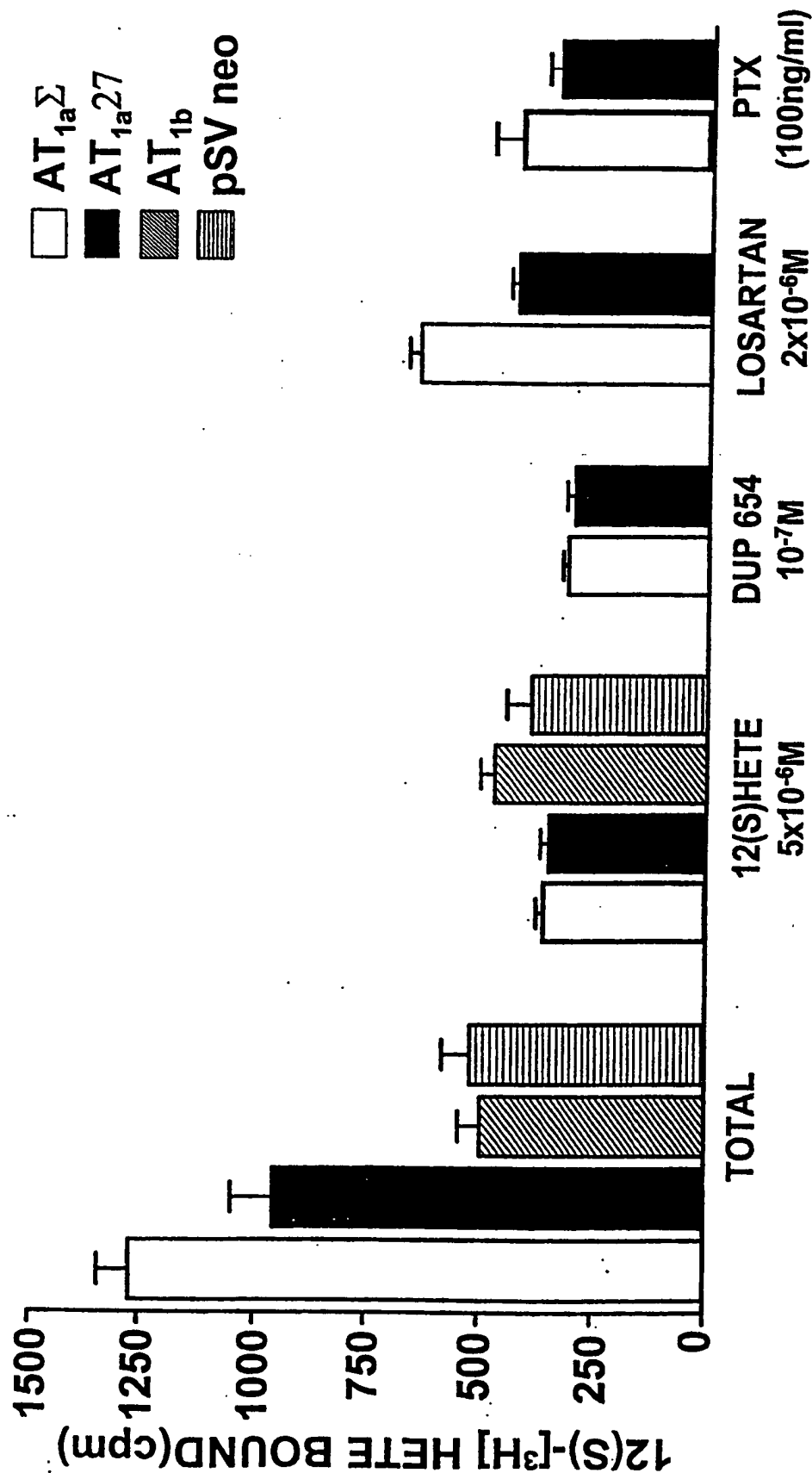


FIGURE 5

## Effect of Losartan(Los) on AII and 12-HETE-induced Mitogenesis in CHO-AT<sub>1a</sub> cells

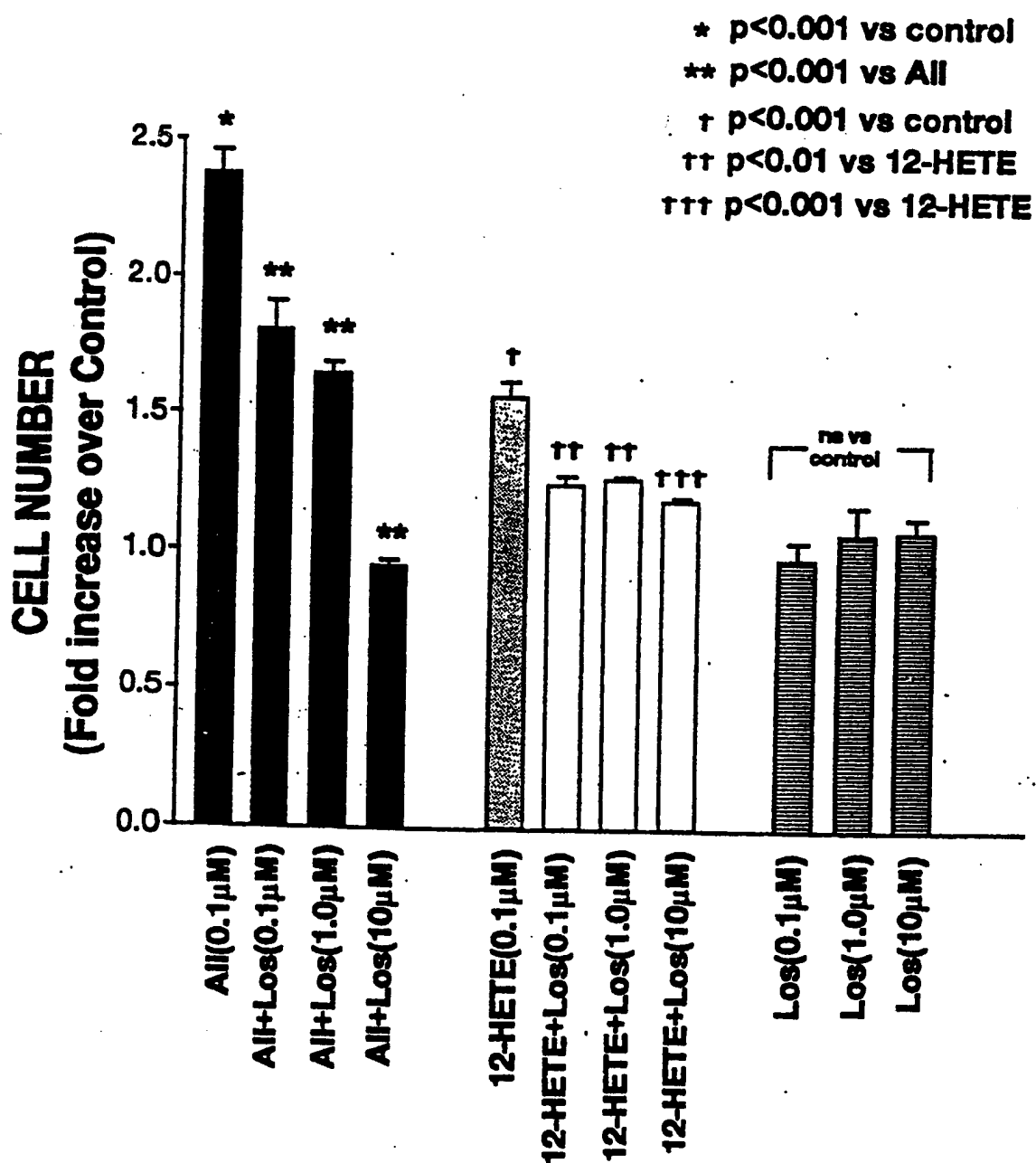


FIGURE 6

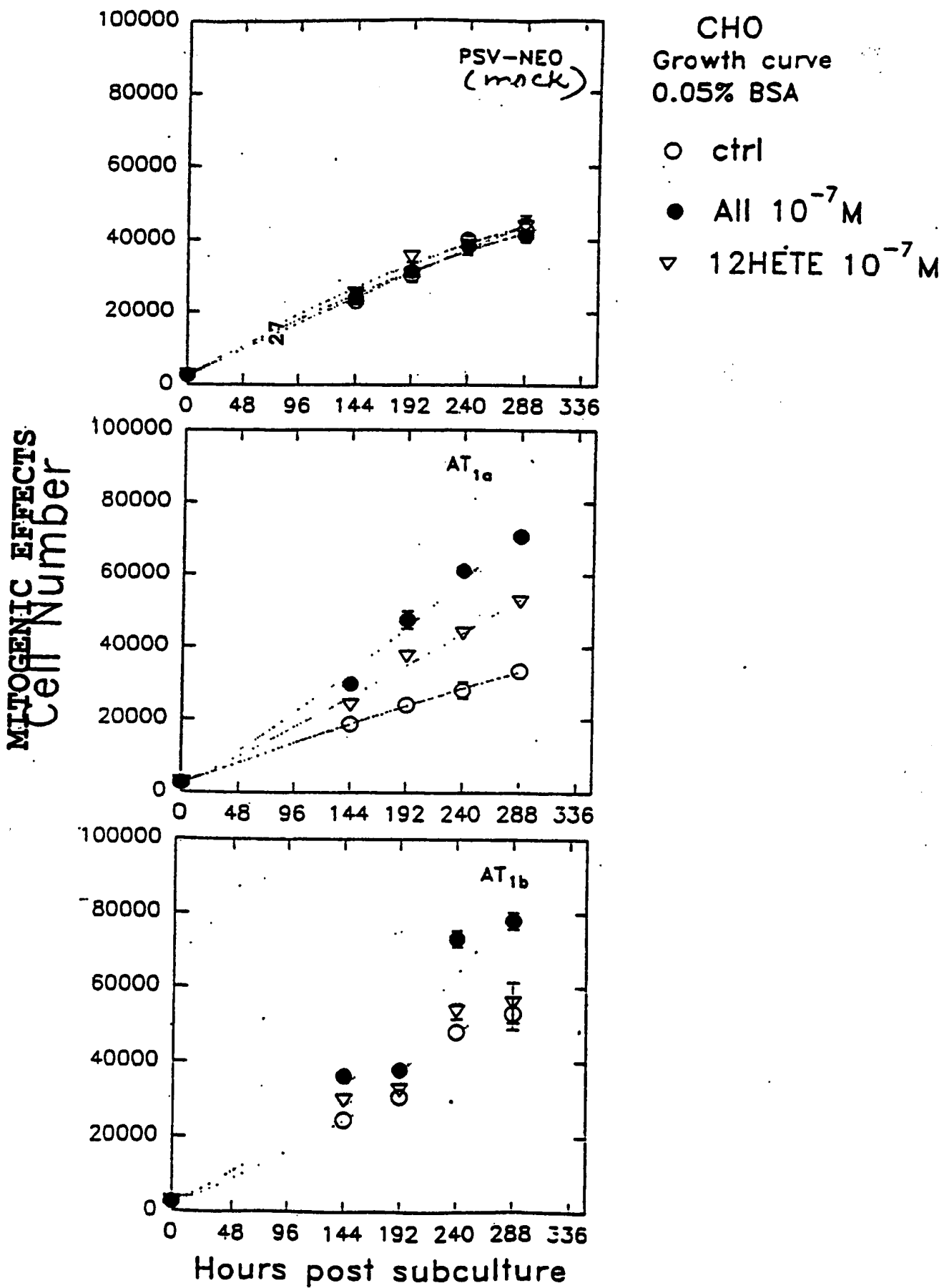




FIGURE 7

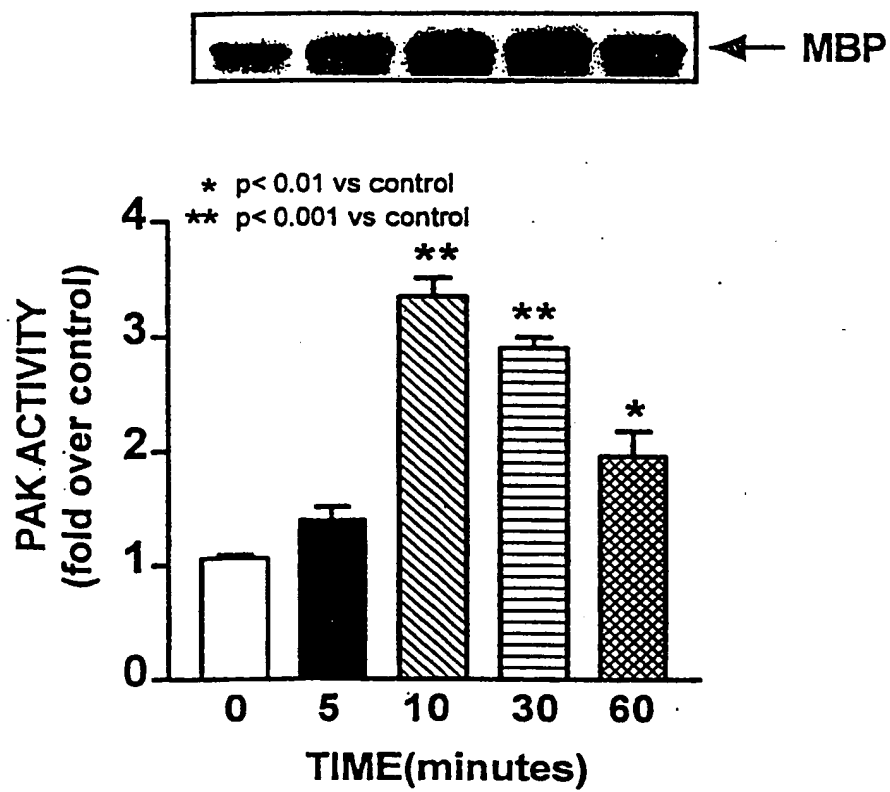


FIGURE 8

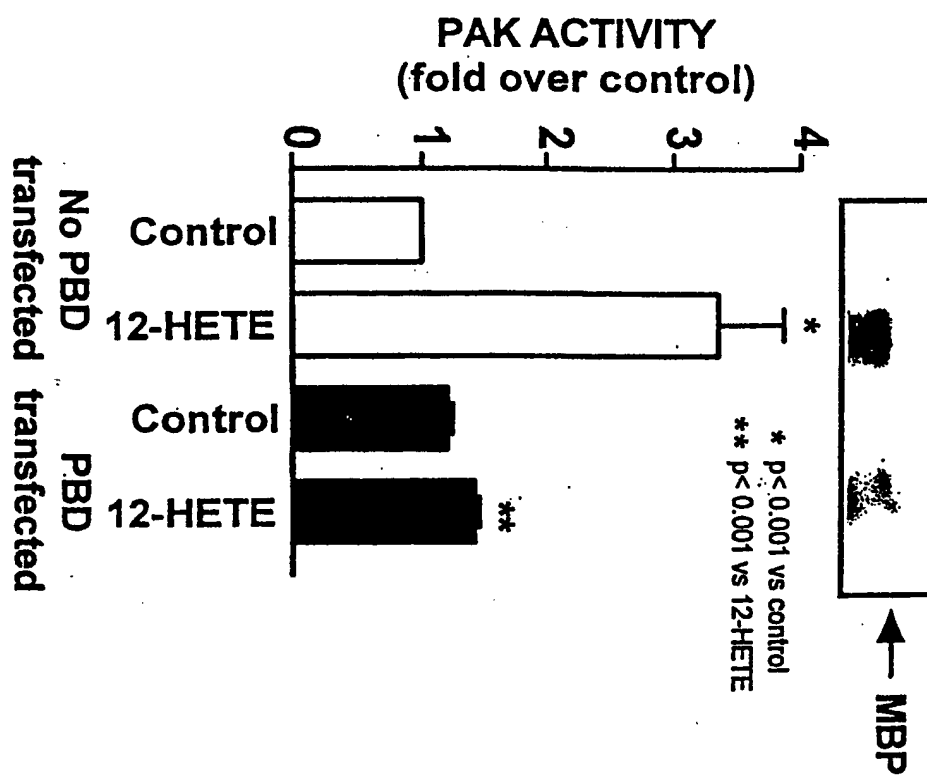
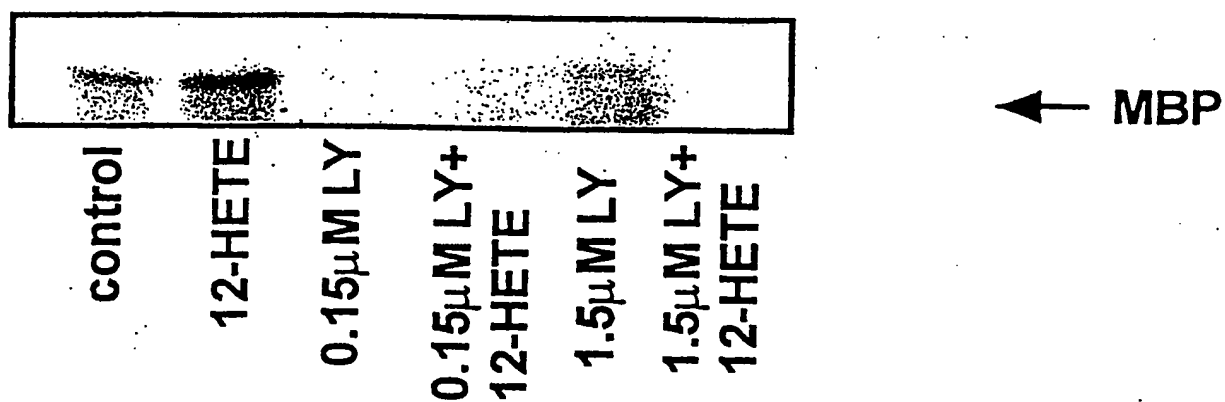


FIGURE 9



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/21570

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K31/415 A61K38/16 A61K31/20 A61K39/395 A61K31/05

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 96 34943 A (HOPE CITY ;NADLER JERRY L (US); NATARAJAN RAMA (US)) 7 November 1996  see page 3, line 28 - page 5, line 29  see page 23, line 9 - page 24, line 29  see page 26, line 32 - page 27, line 27  see page 31, line 24 - line 27  see page 32, line 10 - line 16  see page 43, line 20 - line 22  see page 49, line 33 - line 34  see claims 6,21,25,29-31</p> <p style="text-align: center;">-/--</p>	1-22

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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Authorized officer

Stein, A

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/21570

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARENBERGER, PETR ET AL: "The lipoxygenase inhibitor 2-phenylmethyl-1-naphthol (DuP 654) is a 12(S)-hydroxyeicosatetraenoic acid receptor antagonist in the human epidermal cell line SCL-II" SKIN PHARMACOL. (1993), 6(2), 148-51 , XP002093788 cited in the application see the whole document	1,2, 4-10,15, 17-22
Y	-----	3,11
Y	BLEICH D ET AL: "The stress-activated c-jun protein kinase (JNK) is stimulated by lipoxygenase pathway product 12-HETE in RIN m5F cells" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATION, vol. 230, 1997, pages 448-451, XP002093789 cited in the application see the whole document	3,11
X	WEN Y ET AL: "Mechanisms of ANGII-induced mitogenic responses: role of 12-lipoxygenase and biphasic MAP kinase" AM.J.PHYSIOLOGY:CELL PHYSIOLOGY, vol. 40, no. 4, October 1996, pages c1212-c1220, XP002093790 cited in the application see the whole document	1-9,14, 18-22
A	NATARAJAN R ET AL: "MECHANISM OF ANGIOTENSIN II-INDUCED PROLIFERATION IN BOVINE ADRENOCORTICAL CELLS" ENDOCRINOLOGY, vol. 131, no. 3, September 1992, pages 1174-1180, XP000601236 see the whole document	1-15,21, 22

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/21570

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 1-22  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
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covers only those claims for which fees were paid, specifically claims Nos.:
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restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/21570

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9634943 A	07-11-1996	AU 5637796 A	21-11-1996
		CA 2220156 A	07-11-1996
		EP 0824583 A	25-02-1998







INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification n <sup>6</sup> : <b>A61K 31/415, 38/16, 31/20, 39/395, 31/05</b>		<b>A1</b>	(11) International Publication Number: <b>WO 99/18956</b>
			(43) International Publication Date: 22 April 1999 (22.04.99)
<p>(21) International Application Number: PCT/US98/21570</p> <p>(22) International Filing Date: 14 October 1998 (14.10.98)</p> <p>(30) Priority Data: 60/062,335 15 October 1997 (15.10.97) US</p> <p>(71) Applicant: CITY OF HOPE [US/US]; 1500 East Duarte Road, Duarte, CA 91010-0269 (US).</p> <p>(72) Inventors: NATARAJAN, Rama, Devi; 16837 East Dawn Haven Road, Hacienda Heights, CA 91745 (US). NADLER, Jerry, L.; 2445 Upper Terrace Road, La Crescenta, CA 91214 (US).</p> <p>(74) Agents: FIGG, E., Anthony et al.; Rothwell, Figg, Ernst &amp; Kurz, Suite 701 East, Columbia Square, 555 13th Street N.W., Washington, DC 20004 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: 12(S)-HETE RECEPTOR BLOCKERS</p> <p>(57) Abstract</p> <p>The 12-lipoxygenase product, 12(S)-HETE, mediates hyperproliferative and hyperplastic responses seen in atherosclerosis, diabetes, Parkinson's disease, Alzheimer's, stroke-induced nerve damage and cancer. 12-HETE also mediates inflammation and cell death in some cell systems, particularly B-islet cells of the pancreas. The present invention involves amelioration of disease states mediated by 12(S)-HETE by blocking specific 12(S)-HETE receptors.</p>			

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**12(S)-HETE RECEPTOR BLOCKERS**

This application claims priority from provisional application 60/062,335, filed October 15, 1997.

**STATEMENT REGARDING FEDERALLY SUPPORTED RESEARCH**

5        This invention was made with government support under Grant No. DK 39721 awarded by the National Institutes of Health (NIDDK). The government may have certain rights in the invention.

**BACKGROUND OF THE INVENTION**

10    **Field of the Invention**

      The present invention relates to blockade of the 12(S)-HETE cell surface receptor as a treatment for conditions of the body which result from stimulation or overstimulation of the receptor. 12(S)-HETE, a product  
15 of the 12-lipoxygenase pathway, mediates the hyperproliferative and inflammatory responses present in such diseases as atherosclerosis, psoriasis, diabetes, and cancer. 12(S)-HETE also mediates inflammatory responses and cell death in some cell  
20 types, particularly pancreatic islet beta cells and nerve cells. Blockade of the 12(S)-HETE receptor

ameliorates the symptoms and arrests the mitogenic cellular responses.

### **Background**

Lipoxygenases (LO) are metabolic enzymes which  
5 catalyze the stereospecific oxygenation of  
polyunsaturated fatty acids to hydroperoxy fatty acids  
(Hamberg et al., J. Biol. Chem. 242:5329-5335 (1967)).  
The physiological function of 12-LO, the mammalian  
enzyme which catalyzes the oxygenation of arachidonic  
10 acid to (S)-12-hydroperoxyeicosatetraenoic acid (12-  
HPETE) and (S)-12-hydroxyeicosatetraenoic acid (12(S)-  
HETE), is unclear.

12-LO exists as two isoforms which are the  
products of different genes, leukocyte-type 12-LO and  
15 platelet-type 12-LO, which share 65% homology at the  
amino acid level (Izumi et al., Proc. Natl. Acad. Sci.,  
USA 87:7477-7481 (1990); Funk et al., Proc. Natl. Acad.  
Sci. USA 87:5638-5642 (1990)). The products of the 12-  
LO pathway, such as 12(S)-HETE, have been shown to play  
20 important roles in diseases such as atherosclerosis,  
diabetes, and cancer.

12(S)-HETE has direct mitogenic and hypertrophic  
effects in vascular cells. It is also a potent

chemoattractant for vascular smooth muscle cells (VSMC) and can activate oncogenes such as c-fos and ras and key growth-related kinases such as mitogen-activated protein kinases (ERK, JNK, PAK, p38) and protein kinase C. New results also indicate that 12(S)-HETE can directly increase monocyte binding. Human aortic endothelial cells incubated with 12(S)-HETE for four hours prior to monocyte adhesion assays resulted in an average increase of 3-fold (range of 1.5-5 fold) in monocyte binding as compared to untreated cells. In addition, glucose-induced monocyte adhesion was abrogated by the inhibition of 12-LO using both phenidone, a non-specific LO inhibitor, and baicalein, a more specific 12-LO inhibitor. The adhesion caused by 12-LO products appears to be monocyte-specific.

The 12-LO pathway is activated in pancreatic islets by cytokines and may participate in islet cell destruction. In inflammatory diseases, this pathway plays crucial roles in transmitting distinctive signals within the cell. Using inhibitors of the 12-LO enzyme pathway, researchers have been able to prevent inflammation and cellular damage. Furthermore, VSMC cultured under high glucose (HG) conditions produce increased amounts of 12(S)-HETE (Natarajan et al.,

Proc. Natl. Acad. Sci. USA 90:4947-4951 (1993). Thus, this pathway may be key to the accelerated cardiovascular disease observed in diabetes.

The LO pathway also plays a role in the growth-promoting effects of angiotensin II (AII) and in the chemotactic effects of platelet-derived growth factor: the products of the 12-LO pathway, and 12(S)-HETE in particular, are associated with the hypertrophic, hyperplastic, and mitogenic effects induced by AII.

10 Wen et al., 271 Am. J. Physiol. (40 Cell Physiol.) C1212-C1220 (1996); (Natarajan et al., Hypertension 23:1142-1147 (1994)). The proliferative effects of AII are inhibited by baicalein, a LO inhibitor. The mitogenic effects of 12(S)-HETE are similar to those of

15 AII and are abrogated by pertussis toxin, implicating a G-protein mechanism. The 12-LO enzyme pathway is known to generate proinflammatory mediators in a variety of cells (O.R. Etingin et al., J. Lipid Res. 31:299-305 (1990); V.A. Folcik and M.K. Cathcart J. Lipid Res.

20 34:69-79 (1993)). Human and rat pancreatic B-cells specifically express active leukocyte type 12-LO (V.P. Shannon et al., Am. J. Physiol. 263:E828-E836 (1992); D.S. Bleich et al., Endocrinol. 136:5736-5744 (1995)). Recent evidence implicates products of the 12-LO

pathway in nerve cell death associated with Parkinson's disease, Alzheimer's disease and other inflammatory nerve cell conditions (Neuron 19:453-463 (1997)).

Because 12(S)-HETE has several biological effects  
5 linked to cellular growth in vascular smooth muscle and cardiac fibroblasts (Natarajan et al., Hypertension 23:1142-1147 (1994); Wen et al., Am. J. Physiol. 271:C1212-C1220 (1996)), it is implicated in the etiology of cardiovascular disease. Further evidence  
10 that 12(S)-HETE is responsible for the cellular responses seen in cardiovascular disease in diabetic patients includes the fact that monocyte binding to cultured human aortic endothelial cells increases in chronic high glucose conditions, and that this is  
15 coincident with increased formation of LO products such as 12(S)-HETE. (Kim et al., Diabetes 43:1103-1107 (1994)). Furthermore, treatment of aortic endothelial cells with 12(S)-HETE increases monocyte binding, likely by stimulating JNK activity and inducing CS-1.  
20 12(S)-HETE can also stimulate vascular endothelial growth factor (VEGF) gene expression in vascular smooth muscle (Am. J. Physiol. 273: H2224-H2231 (1997)). VEGF has been linked to angiogenesis in diabetic

retinopathy, tumor growth and atherosclerotic vascular disease.

12(S)-HETE is also regarded as a mediator of inflammation and hyperproliferation of the skin

5 (Arenberger et al., Skin Pharmacol. 6:148-151 (1993); Gross et al., J. Invest. Dermatol. 94:446-451 (1990)) and is therefore implicated in skin diseases.

12(S)-HETE has been shown to enhance tumor cell adhesion to endothelial cells. (Honn et al., Cancer  
10 Metastasis Rev. 13:365-396 (1994)). 12(S)-HETE can directly increase p21 activated kinase (PAK). The effect appears to be through activation of small GTP binding proteins such as RAC and through activation of PI3K.

15 The precise mechanisms of 12(S)-HETE action are not clear, however recent studies have shown that the LO product, 12(S)-HETE, activates c-jun amino terminal kinase (JNK) (Wen et al., Circ. Res. 81:651-655  
(1997)). JNK is a small GTP-binding protein and a  
20 member of the MAP kinase family which is involved in cellular growth, inflammation, and apoptosis (Force et al., Circ. Res. 78:947-953 (1994)) and in cell cycle progression through G<sub>1</sub> (Olson et al., Science 269:1270-1272 (1995)). Evidence shows that JNK can serve as a



positive or negative modulator of cell growth in different cells. Olson et al., 269 Science 1270-1272 (1995); Yan et al., 372 Nature 798-800 (1994). 12(S)-HETE activation of JNK may also be the mediator of cytokine-induced pancreatic B-cell damage (Bleich et al., Biochem. Biophys. Res. Commun. 230:448-451 (1997)).

Newer evidence indicates that the growth factor and potent vasoconstrictor AII, linked to type-1 receptor activation, can activate JNK and PAK (Wen et al., Circ. Res. 81:651-655 (1997); Schmitz et al., Circ. Res. 82:1272-1278 (1998)). Furthermore, AII can modulate serum deprivation-induced apoptosis by increasing JNK activity in vascular smooth muscle cells, Sueror et al., Circulation, Supp. 1, I-281 (1994), mediated by lipids derived from the 12-LO pathway, such as 12(S)-HETE. This indicates that 12-LO products participate in JNK activation at least in part through G<sub>i</sub>-protein signaling. The ability of pertussis toxin to block the activation of JNK by 12(S)-HETE also supports the theory that 12(S)-HETE is a mediator of AII-induced JNK activation through a G<sub>i</sub>-mediated pathway.

While several studies have demonstrated the potent biological effects of lipoxxygenase products, the mechanisms of action of these effects are not known. Some reports have hinted at the presence of 12(S)-HETE  
5 receptors on transformed cells. Binding sites for 12(S)-HETE have been detected in carcinoma cells (Herbertsson and Hammarstrom, FEBS 298:249-252 (1992), on melanoma cells (Liu et al., Proc. Natl. Acad. Sci. USA 92:9323-9327 (1995), and in a human epidermal cell  
10 line (Gross et al., J. Invest. Dermatol. 94:446-451 (1990); Suss et al., Exptl. Cell Res. 191(2):204-208 (1990)).

The 12(S)-HETE receptors described in carcinoma cells are cytosolic receptors (Herbertsson and  
15 Hammarstrom, Biochem. Biophys. Acta 1244:191-197 (1995)), activation of which may mediate 12(S)-HETE induced mRNA production of genes coding for the integrin  $\alpha_{IIb}\beta_3$  (Chang et al., Biochem. Biophys. Res. Comm. 176:108-113 (1991)). The localization of this  
20 receptor is different from plasma cell membrane receptors coupled to a G-protein and acting through second messengers.

12(S)-HETE receptors on the cell surface of murine melanoma cells have been described. These receptors

stimulate the second messengers diacylglycerol and inositol phosphate, via a G-protein mechanism, resulting in protein kinase C<sub>2</sub> activation. (Liu et al., Proc. Natl. Acad. Sci. USA 92:9323-9327 (1995)). The binding  
5 of 12(S)-HETE to these receptors was blocked by 13(s)-hydroxyoctadecadienoic acid, a LO metabolite of linoleic acid, ablating the 12(S)-HETE increased adhesion of the cells to fibronectin. These authors suggest 12(S)-HETE may act in a "cytokine" fashion to  
10 regulate responses of adjacent tumor cells, endothelial cells, and platelets.

Receptors for 15-HETE have been identified in mast/basophil (PT-18) cells and were shown to possess properties of G-protein-coupled receptors (Vonakis and  
15 Vanderhoek, J. Biol. Chem. 267:23625-23631 (1992)). Specific binding of 15-HETE to these receptors stimulated 5-LO, and while 12(S)-HETE was found to be an effective competitor of [<sup>3</sup>H]15-HETE binding to PT-18 cells, suggesting that 12(S)-HETE binds to the specific  
20 15-HETE receptor, the binding of 12(S)-HETE did not stimulate the lipxygenase. Very recent studies have indicated the activation of a cell surface G-protein-coupled 5-HETE receptor in neutrophils (Capadici et al., J. Clin. Invest. 102:165-175 (1998)).

The high affinity 12(S)-HETE-specific receptors in a human epidermal carcinoma cell line were induced by  $\gamma$ -IFN (Gross et al., J. Invest. Dermatol. 94:446-451 (1990)). Saturation binding of 12(S)-HETE to these  
5 receptors did not stimulate cell growth, therefore, the function of these receptors in the skin is entirely speculative, and not related to the AII-induced cellular effects mediated by cell surface 12(S)-HETE receptors in fibroblasts overexpressing the AII  
10 receptor and potentially in vascular smooth muscle cells. Two recent studies have indicated two additional agents which could reduce 12(S)-HETE binding (Kemeny and Ruzicka, Agents Actions 32:339-342 (1991);  
Kemeny et al., Arch. Dermatol. Res. 283:333-336  
15 (1991)).

Specific inhibitors of 12-LO have been described. Gorins et al., J. Med. Chem. 39:4871-4878 (1996). In that study, a series of substituted (carboxyalkyl)benzyl ethers were found to be selective  
20 inhibitors of leukocyte-type 12-LO. These inhibitors of 12-LO acted by serving as structural analogs for the enzyme. Gorins et al., J. Med. Chem. 39:4871-4878 (1996). The 5-LO inhibitor, 2-phenylmethyl-1-naphthol (DuP654), has also been shown to specifically inhibit

binding of 12(S)-HETE to receptors on the human epidermal cell line SCL-II. Arenberger et al., Skin Pharmacol 6:148-151 1993).

5        In vivo, inhibition of 12-LO has lowered blood pressure in several models of hypertensive animals, including rats (Stern et al., Am. J. Physiol. 257:H434-H443 (1989); Nozawa et al., Am. J. Physiol. 259:H1447-H1780 (1990)). In addition, blockage of 12-LO activity has alleviated the growth-factor induced effects of 10        12-HETE in vascular cells. This, along with the known increased expression of 12-LO observed in animal models of diabetes (Gu et al., Am. Diabet. Assoc. Meeting (1996); Natarajan et al., Intl. Aldosterone Meeting (1998)) and diabetes induced accelerated 15        atherosclerosis (Gerrity et al., Circulation 1175 (1997)) strongly implicate 12-HETE and the 12-LO pathway in the etiology of these diseases. The harmful effects of 12-LO activation are ameliorated by blocking the production of 12(S)-HETE, providing the rationale 20        for a method of treatment which focusses on preventing 12(S)-HETE binding to its receptor.

There is currently no inhibitor of 12(S)-HETE receptor binding in clinical use. Due to the existence of several isoforms of 12-LO, blockage of the 12-HETE

receptor is a more specific and direct way to correct a disease state in which there is increased production of 12(S)-HETE or the receptors are up-regulated. This invention therefore, could provide the basis for the development of interventions to reduce cardiovascular disease, diabetes, and cancer.

#### SUMMARY OF THE INVENTION

The present invention relates to a method of inhibiting the effects of the LO product 12(S)-HETE by blocking 12(S)-HETE receptors comprising the administration of an effective amount of a 12(S)-HETE receptor antagonist or an antibody directed against a cell surface 12(S)-HETE receptor. The blockade of 12(S)-HETE receptors provides a means for ameliorating the proliferative and mitogenic effects of glucose, PDGF or AII-induced 12(S)-HETE production, or direct effects of 12(S)-HETE inflammatory actions.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the binding of tritiated 12(S)-HETE and DuP654 to CHO-AT<sub>1a</sub> cells at increasing concentrations of unlabeled 12(S)-HETE.

Figure 2 shows a competitive binding curve of tritiated and unlabeled 12(S)-HETE with CHO-AT<sub>1a</sub> cells. Figure 3 shows the effect of the 12(S)-HETE receptor antagonist, DuP654, on AII- and 12(S)-HETE-induced growth in CHO-AT<sub>1a</sub> cells.

Figure 4 shows the effect of three agents which bind to CHO-AT<sub>1a</sub> cells, DuP654 (a 12(s)HETE receptor antagonist), Losartan (a specific AII<sub>1a</sub> receptor antagonist) and pertussis toxin, relative to 12(S)-HETE effects. Inhibition of labeled 12(S)-HETE binding sites on AT<sub>1a</sub>Δ and AT<sub>1a</sub>27 (2 cloned overexpressing AT<sub>1a</sub>) cells are shown and pSV neo mock transfected cells.

Figure 5 shows the effect of Losartan on AII and 12(S)-HETE-induced mitogenesis in CHO-AT<sub>1a</sub> cells.

Figure 6 shows the mitogenic effects of AII and 12(S)-HETE on Psv neo mock transfected cells, AT<sub>1a</sub> expressing cells and AT<sub>1b</sub> expressing cells.

Figure 7 shows the time course of PAK activation by 12(S)-HETE (10<sup>-7</sup> M) in CHO-AT<sub>1a</sub> cells.

Figure 8 shows the inhibitory effect of transient transfection of CHO-AT<sub>1a</sub> cells by a PBD plasmid on 12(S)-HETE-induced PAK activation.

Figure 9 is a representative autoradiogram of phosphorylated MBP bands demonstrating inhibition of

12(S)-HETE induced PAK activity by the PI 3-kinase inhibitor, LY294002.

#### DETAILED DESCRIPTION OF THE INVENTION

Angiotensin II (AII) has been shown to stimulate,  
5 through the AII AT<sub>1</sub> receptor, 12-LO activity in murine  
macrophages, Scheidegger et al., J. Biol. Chem.  
272:21609-21615 (1997), and in smooth muscle cells,  
Natarajan et al., Proc. Natl. Acad. Sci., USA 90:4947-  
4951 (1993); Kim et al., Atherosclerol. Thromb. Vasc.  
10 Biol. 15:942-948 (1995). Stimulation of the 12-LO  
pathway in murine macrophages resulted in an increase  
of monocyte chemotaxis (Scheidegger et al., (in press,  
1997)), presumably through modification of LDL. This  
activity links AII activation of 12-LO to  
15 atherosclerotic disease.

The potential mechanisms of AII-induced mitogenic  
effects in a Chinese hamster ovary fibroblast cell line  
overexpressing the rat vascular type 1a AII (AT<sub>1a</sub>)  
receptor have recently been examined. See Wen et al.,  
20 Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220  
(1996). AII had mitogenic effects in these cells,  
leading to a sustained increase in DNA synthesis as  
well as cell number. It was also observed in these



cells that the 12-lipoxygenase product, 12(S)-HETE, also had direct mitogenic effects in these cells. See Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996). Furthermore, 12(S)-HETE did not have any mitogenic effects in mock transfected cells. The addition of 12(S)-HETE to these CHO-AT<sub>1a</sub> cells led to a significant increase in the activity of the key growth-related kinases, mitogen activated protein kinase (Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996)), and c-jun amino terminal kinase (Wen et al., Circ. Res. 81:651-655 (1997)). This work has suggested that over expression of the AT<sub>1a</sub> receptor plays a role in inducing a putative 12(S)-HETE receptor, which is supported by the observation that the mitogenic effects of 12(S)-HETE were completely abrogated by pretreatment of the cells with pertussis toxin. Thus, the effects of 12(S)-HETE may be mediated by a Gi protein-coupled receptor. See example 3, below. Application of AII to CHO-AT<sub>1a</sub> cells resulted in a 2-fold increase in 12(S)-HETE formation and cell proliferation. These proliferative effects were inhibited by the 12-LO inhibitor, baicalein.

In accordance with the present invention, a 12-HETE receptor has been discovered and characterized.

For the first time, a specific high affinity 12(S)-HETE receptor has been identified. Chinese hamster ovary (CHO) fibroblasts that stably overexpress the rat vascular angiotensin type 1a receptor (CHO-AT<sub>1a</sub>) have been found to carry this receptor. This receptor is not present in mock transfected cells. Experiments have been performed which indicate that this receptor has characteristics of a G-protein coupled receptor. Furthermore, there is evidence of crosstalk between this receptor and the AT<sub>1b</sub> receptor, since a specific antagonist, Losartan, was able to partially block the binding of 12(S)-HETE to the cells and also blocked the mitogenic effects of 12(S)-HETE. Furthermore, a 12(S)-HETE receptor antagonist blocked 12(S)-HETE mitogenic effects and partially blocked AII mitogenic effects. Increased actions of vasoactive and growth promoting agents, such as angiotensin II, under pathologic conditions may up-regulate 12(S)-HETE receptors. Hence, further studies of this receptor in vascular and other cells, as well as the development of specific receptor antagonists, are expected to be therapeutically important.

It has also been found that hyperglycemic conditions result in both increased monocyte binding to

human aortic endothelial cells (HAEC) and increased 12(S)-HETE and 15-HETE activity. Neutrophil binding is not increased. In HAEC incubated in vitro with 12-LO products, increased monocyte binding, JNK activation, and induction of CS-1 fibronectin were detected, suggesting that the upregulation of 12-LO activity seen in hyperglycemia may exacerbate atherosclerosis by stimulating adhesion of monocytes through JNK activation and CS-1 production. For example, monocytes inabated with  $10^{-7}$  M 12(S)-HETE for 12 minutes at room temperature or at 37°C prior to monocyte adhesion assay demonstrated increased adhesion over untreated cells. See Example 10.

Blockade of 12(S)-HETE receptor binding therefore is a new method of treating disorders associated with increased 12-lipoxygenase expression and activity. These diseases include atherosclerotic cardiovascular disease, glucose and diabetes-induced complications, cytokine-induced inflammatory cellular effects, and tumor cell growth and metastasis.

The kinetics of radioactive [ $^3\text{H}$ ]12(S)-HETE binding to these cells at 4°C have been examined. These studies have revealed the presence of specific high affinity binding sites for 12(S)-HETE on these cells.

Specificity was determined by the observation that this binding of tritiated 12(S)-HETE was displaced by unlabeled 12(S)-HETE. A one site fit model yielded a K<sub>d</sub> of 38.4 nM. See Example 1. The binding kinetics of [3H]12(S)-HETE have revealed the presence of specific high affinity 12(S)-HETE binding sites on CHO-AT<sub>1</sub> cells, but not in mock transfected cells; these results suggest that AII-induced mitogenic effects involve the production of reactive oxygen species and LO products via activation of G-protein-coupled receptors.

DuP654 could completely inhibit 12(S)-HETE-induced mitogenic effects. DuP654 significantly reduced cell growth induced by either AII or 12(S)-HETE at a concentration of 0.1 μM. Tritiated 12(S)-HETE binding was also blocked by pertussis toxin (Figure 3). Pertussis toxin has been shown to ablate 12(S)-HETE-induced mitogenic effects (Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996)), implicating the involvement of a G<sub>i</sub> protein-coupled receptor. Losartan, a specific angiotensin AT<sub>1a</sub> receptor antagonist now in clinical use for the treatment of hypertension, partially blocked tritiated 12(S)-HETE binding (Figure 4). Similarly, it partially blocked 12(S)-HETE-induced mitogenic effects in these

CHO-AT<sub>1a</sub> cells, while fully inhibiting AII-induced proliferative effects (Figure 5). 12(S)-HETE had mitogenic effects only in CHO-AT<sub>1a</sub> cells, but not in mock transfected cells (pSVneo), nor in CHO cells overexpressing the angiotensin AT<sub>1b</sub> receptor (Figure 6).

This invention involves a method for inhibiting the effects of 12(S)-HETE by administration of an effective amount of a 12(S)-HETE receptor antagonist. The method is useful for the treatment or prophylaxis of conditions in which 12(S)-HETE receptor activation contributes to adverse effects. For example, the method of this invention may be employed for the treatment or prophylaxis of atherosclerotic cardiovascular disease, glucose-induced complications of diabetes, cytokine-induced inflammatory diseases and tumor cell growth and metastasis.

The 12(S)-HETE receptor antagonist may be any agent that blocks or significantly inhibits binding of 12(S)-HETE to its receptor. Such agents include DuP654 (2-phenylmethyl-1-naphthol), Losartan (2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole, potassium salt), pertussis toxin, 12(S)-HETE analogs, peptides and

peptide analogs having affinity for the binding site on the 12(S)-HETE receptor (especially antibodies which can block 12(S)-HETE receptors), antibodies to the 12(S)-HETE receptor, and the like.

5           The determination of appropriate, well-tolerated dosage forms for administration to humans for use in the present invention is within the ordinary skill in the art. Such dosage forms include tablets, capsules, syrups, suspensions, drops, injectable solutions,  
10           lozenges, implants, transdermal patches, and other dosage forms well known in the art for enteral or parenteral administration. Based on in vitro experiments on the effect of 12(S)-HETE blocking drugs on 12(S)-HETE binding, a dose of between about 0.5 and  
15           about 30 mg/kg/day would be effective in blocking 12(S)-HETE receptors in humans in vivo, and preferably from about 1 to about 10 mg/kg/day.

          The present invention is further illustrated by the following examples, which are not intended to be  
20           limiting.

EXAMPLESExample 1

Kinetics of [ $^3\text{H}$ ]12(S)-HETE binding to CHO-AT<sub>1a</sub> cells. Figures 1 and 2 are a competition curves which  
5 examine the specificity of [ $^3\text{H}$ ]12(S)-HETE binding. CHO-AT<sub>1a</sub> cells were grown to confluence in 24 well tissue culture dishes in HAM's F12 medium containing 10% fetal calf serum. The cells were then rinsed and placed in fresh medium, HAM's F12/HEPES with no other additives  
10 (450 ul per well). Serial dilutions of unlabeled 12(S)-HETE or DuP654 were added to the wells. Commercial unlabeled 12(S)-HETE (BioMol Corp.) was dried and reconstituted in ethanol to obtain a stock solution of 5 mM. DuP654 was dissolved in DMSO to get  
15 a stock solution of 5 mM. These were then serially diluted and added in a volume of 1 ul to the wells to obtain the final concentrations indicated. Then [ $^3\text{H}$ ]12(S)-HETE (10,000 cpm in a volume of 50 ul per well) was added from a stock solution obtained by  
20 adding the tracer to the medium. The plates were then incubated at 4°C with continuous shaking for 2 hr. The cells were then washed 2 times with cold PBS and lysed in 0.3N NaOH (200 ul). Radioactivity in the cell lysates was quantitated in scintillation counter.

Affinities and binding constants were obtained using Matlab computer software (Mlab, Civilized Software Inc., Bethesda, MD).

This experiment revealed the presence of specific high affinity binding sites for 12(S)-HETE on these cells. A one site fit model yielded a  $K_d$  of 38.4 nM. Specificity was determined by the observation that this binding of [ $^3H$ ]12(S)-HETE was displaced by unlabeled 12(S)-HETE.

10

#### Example 2

Reduction of cell growth induced by AII or 12(S)-HETE.

DuP654 significantly reduced cell growth induced by either AII or 12(S)-HETE at a concentration of 0.1  $\mu$ M. Complete inhibition of 12(S)-HETE induced mitogenic effects was seen. See Figure 3.

15

#### Example 3

Blockade of the 12(S)-HETE receptor by a specific antagonist.

20

Tritrated 12(S)-HETE binding is blocked by unlabeled 12(S)-HETE. See Figure 4. DuP654, a 12(S)-HETE receptor antagonist, was shown also to block 12(S)-HETE at a concentration of 0.1  $\mu$ M in both AT<sub>1a</sub> $\Sigma$  and AT<sub>1a</sub>27 cell types, two clones of CHO cells which



overexpress the AII<sub>1a</sub> receptor. See Figure 4. The cells were grown as described in Example 1.

#### Example 4

Blockade of 12(S)-HETE binding by pertussis toxin.

5 Cells were grown as described in Example 1. Prior to addition of drug (12(S)-HETE or DuP654) to the cells, the cultures were preincubated in HAM's F12 medium + 0.1% BSA for two hours at 37°C with or without 100 ng/ml pertussis toxin. Serial dilutions of unlabeled  
10 12(S)-HETE or DuP654 were added, followed by [<sup>3</sup>H]12(S)-HETE as described for Example 1. After incubation and washing, radioactivity in the cell lysates was quantitated. See Figure 4. As discussed above, pertussis toxin could also ablate 12(S)-HETE-induced  
15 mitogenic effects. This implicates the involvement of a G<sub>i</sub>-protein-coupled receptor.

#### Example 5

Partial blockade of 12(S)-HETE mitogenesis by the specific angiotensin AT<sub>1a</sub> receptor antagonist, Losartan,  
20 in CHO-AT<sub>1a</sub> cells. (Figure 5)

CHO-AT<sub>1a</sub> cells were plated in 12-well dishes (about 5-10,000 cells per well) for 24 hr. in growth medium consisting of HAM's, F12 + 10% FCS. They were then serum depleted for 72 hours by replacing the medium

with HAM's F12 + 0.1% BSA. This medium was then freshly replaced along with AII or 12-HETE (0.1  $\mu$ M each) prior to addition of drug to the cells. Losartan was added as a solution in water to the cells 15 min. prior to the addition of AII or 12-HETE. The final concentration of Losartan was as indicated in Figure 4. Fresh medium containing the same concentrations of AII or 12(S)-HETE plus Losartan was replaced every 48 hours. At the end of 8 days, the medium was removed, and 1 ml trypsin was added per well followed by 1 ml isotone after 3 min. These trypsinized cells were counted on a Coulter counter. Losartan partially blocked 12(S)-HETE-induced mitogenic effects and fully blocked AII-induced proliferative effects. See Figure 5). Losartan also partially blocked [ $^3$ H] 12-HETE binding (Figure 4).

#### Example 6

Dependency of 12(S)-HETE mitogenic effects on expression of the AT<sub>1a</sub> receptor.

The three cell lines, CHO-AT<sub>1a</sub>, CHO-AT<sub>1b</sub> and mock transfected CHO cells were gifts from Dr. Eric Clauser (Inserm Unit, Paris, France). These cells were plated in 12 well dishes in HAM's F12 medium + 10% FCS. After 72 hours serum depletion in HAM's F12 + 0.05% FCS, the

cells were treated with AII or 12(S)-HETE (0.1  $\mu$ M). Cells counts (after trypsinization) were taken at 48 hour intervals and fresh medium along with AII or 12-HETE added at these 48 hour intervals. 12(S)-HETE had mitogenic effects only in CHO-AT<sub>1a</sub> cells, but not in mock transfected cells (pSVneo), nor in CHO cells overexpressing the angiotensin AT<sub>1b</sub> receptor. See Figure 6.

#### Example 7

PAK activation by 12(S)-HETE.

CHO-AT<sub>1a</sub> cells were gently washed and placed in depletion medium (HAM's F-12 medium containing 1 mg/ml BSA and 20 mM HEPES, pH 7.4) for 72 hours prior to use. After incubation for 30 minutes, the cells were treated with 10<sup>-7</sup>M 12(S)-HETE or with ethanol. The 12(S)-HETE treatment was terminated by washing twice with PBS and adding 300  $\mu$ l lysis buffer (50 mM HEPES, pH 7.5, containing 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1% NP-40, 2.5% glycerol and 1 mM Na<sub>3</sub>VO<sub>4</sub> containing the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and aprotinin) followed by sedimentation at 14,000 xg at 4 °C for ten minutes. Protein determination was performed by the Bradford method. The top panel shows

a representative autoradiogram of phosphonycated myelin basic protein (MBP) bands from a gel. PAK activity was measured as follows. First, 300 µg of lysate protein was incubated with PAK antibody (1:20) in lysis buffer overnight at 4°C, followed by incubation with 60 µl of a 50% slurry of protein A beads for 60 minutes. After washing three times with lysis buffer and twice with kinase buffer (50mM HEPES, pH 7.4, 10mM MgCl<sub>2</sub>, 10mM MnCl<sub>2</sub>, and 0.2 dithiothreitol) containing 2 µl MBP, 20 µM ATP and 5µCi [γ-<sup>32</sup>P] ATP, the kinase activity was measured in 60 µl kinase buffer. After incubation for 30 minutes at 30°C, the reaction was stopped with 5x Laemmli sample buffer and resolved on a 12% SDS-polyacrylamide gel, followed by autoradiography. The bottom panel shows the densitometric quantitation of PAK activity stimulated with 10<sup>-7</sup> M 12(S)-HETE or ethanol (control) for the time indicated. Each point is an average (mean ± SE) from at least 3 separate experiments. Results are expressed as stimulation over control.

#### Example 8

Inhibition of 12(S)-HETE induced PAK activation by transient transfection by a PAK binding domain (PDB) plasmid.

The degree of 12(S)-HETE induce PAK activation was compared in CHO-AT<sub>1a</sub> cells which had been transiently transfected with a PDB plasmid and cells which had not been transfected.

5 For the PBD-transfected group, CHO-AT<sub>1a</sub> cells were transiently transfected with 15 µg PBD plasmid. For the non-PBD-transfected group, CHO-AT<sub>1a</sub> cells were treated with the same transfection reagents as the PBD-transfected group, but lacking plasmid. Plasmids used  
10 were endotoxin-free and prepared by EndoFree plasmid kit (Qiagen Co.) with the standard protocol. The DNA transfection method used was a cationic liposome-mediated transfection with DOSPER transfection reagent (Boehringer Mannheim Co.) following the manufacturer's  
15 instructions. Briefly, the cells were plated the day before the transfection experiment at  $3 \times 10^6$  cells per 100 mm dish. The next day, cells were washed with Opti-MEM<sup>®</sup> reduced serum medium (Gibco BRL) and incubated in 5 ml of HAM's F-12 medium containing 1%  
20 FBS. Plasmid mixture (45 µg DUSPER/15 µg) was prepared and added to each dish. After a 5 hour incubation, the transfection medium was replaced and 8 ml fresh depletion medium (described in Example 7) continuing 1% FBS for overnight incubation was added. The cells were

washed twice with depletion medium, incubated in the same medium for another 32 hours and harvested. Cells were then treated with  $10^{-7}$ M 12(S)-HETE or ethanol for 10 minutes. The top panel of Figure 8 illustrates a representative autoradiogram of phosphorylated MBP bands from a gel. The bottom panel illustrates the densitometric quantification. Each point is an average (mean  $\pm$  SE) of at least 3 separate experiments. Results are expressed as stimulation over control. The PAK activity was measured as described in Example 7.

#### Example 9

Inhibition of PI 3-kinase by LY294002.

Cells were pretreated with different concentrations of the PI-3 kinase inhibitor, LY294002 or DMSO (control) for 30 minutes, then treated with  $10^{-7}$  M 12(S)-HETE or ethanol (control) for 10 minutes. PAK activity was measured as described in Example 7. Figure 9 shows a representative autoradiogram of phosphorylated MBP bands from 3 similar experiments.

#### Example 10

Increased monocyte adhesion to HAEC by treatment of monocytes with 12(S)-HETE.

Monocytes were incubated with  $10^{-9}$ M 12(S)-HETE for 12 minutes at room temperature (RT) or 37°C or left

untreated at room temperature and assayed for monocytes adhesion. Eight fields were counted for each experiment. Results are presented in Table 1.

Table 1 effect of 12(S)-HETE on Monocyte Adhesion to  
Human Aortic Endothelial Cells

Experiment	NT	12(S)-HETE (RT)	12(S)-HETE (37°C)
1	26.4 ± 12.5	39.6 ± 12.5 <sup>1</sup>	39.0 ± 9.3 <sup>3</sup>
2	28.3 ± 5.0	45.4 ± 7.6 <sup>2</sup>	ND <sup>4</sup>

- 10
1. P = 0.016
  2. P = 0.001
  3. P = 0.01
  4. ND = not done

**CLAIMS**

1. A method for inhibiting the effects of 12-HETE comprising administration of an effective amount of a 12(S)-HETE receptor blocker.
2. The method of claim 1 wherein the receptors are on the cell surface.
3. The method of claim 2 wherein the in the cells are selected from the group consisting of monocytes, endothelial cells, pancreatic islet beta cells, nerve cells, cardiac fibroblasts, cardiac  
5 myocytes and vascular smooth muscle cells.
4. The method of claim 1 wherein the administration of the compound is to an animal.
5. The method of claim 4 wherein the animal is a mammal.
6. The method of claim 4 wherein the animal is a human.
7. The method of claim 1 wherein the blocking of the receptor inhibits 12(S)-HETE binding.
8. The method of claim 1 wherein the blocking of the receptor inhibits receptor activation.
9. The method of claim 1 wherein the blocking of the receptor inhibits cell growth.



10. The method of claim 1 wherein the blocking of the receptor inhibits inflammatory cell damage.

11. The method of claim 1 wherein the blocking of the receptor inhibits cell death.

12. The method of claim 1 wherein the blocking of the receptor reduces monocyte adhesion.

13. The method of claim 1 wherein the blocking of the receptor reduces VEGF production.

14. The method of claim 1 wherein the blocking of the receptor reduces PAK activation.

15. The method of claim 1 wherein the 12(S)-HETE receptor blocker is a 12(S)-HETE receptor antagonist.

16. The method of claim 1 wherein the 12(S)-HETE receptor blocker is an antibody.

17. A method of suppressing the activation of 12(S)-HETE receptors comprising the administration of a compound which prevents the binding of endogenous receptor agonists to the receptor.

18. A method for the treatment or prophylaxis of a disease in which 12(S)-HETE receptor activation contributes to adverse effects which comprises administering an effective amount of a 12(S)-HETE  
5 receptor blocker.

19. The method of claim 18, wherein the disease is atherosclerotic cardiovascular disease, glucose-induced complications of diabetes, Parkinson's disease, Alzheimer's disease, stroke-induced nerve damage,  
5 cytokine induced inflammatory disease or tumor cell growth or metastasis.

20. The method of claim 19, wherein the 12-HETE receptor blocker is selected from the group consisting of DuP654, Losartan, pertussis toxin, a 12(S)-HETE analog, an antibody to the 12(S)-HETE receptor, a  
5 peptide which binds to the 12(S)-HETE receptor and a peptide analog which binds to the 12(S)-HETE receptor.

21. The method of claim 18, wherein the 12-HETE receptor blocker is administered in a dose of from about 0.5 to about 300 mg/kg/day.

22. The method of claim 21, wherein the 12-HETE receptor blocker is administered in a dose of from about 1 to about 10 mg/kg/day.

FIG. 1

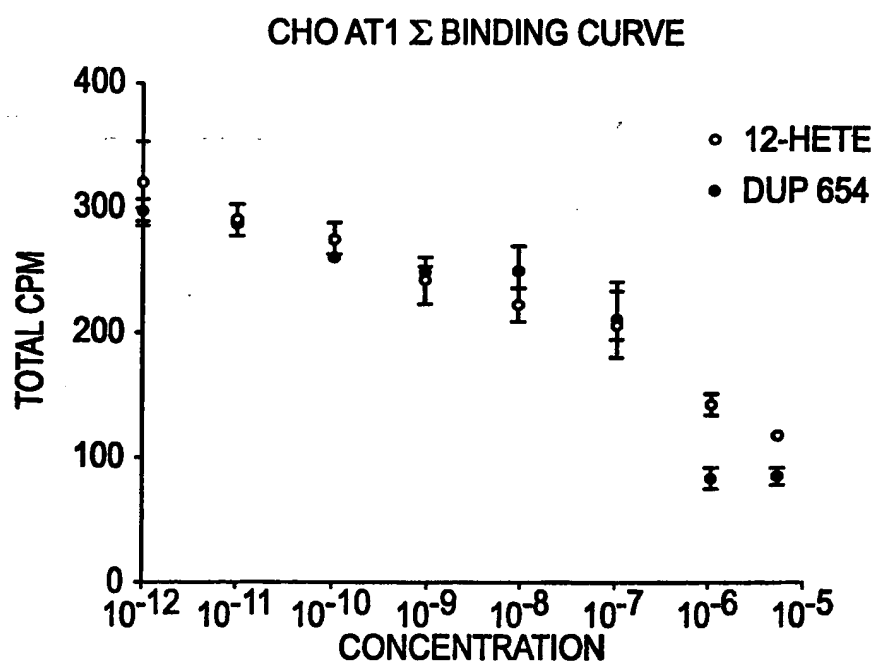
 $[^3\text{H}]12(\text{S})\text{-HETE}$  BINDING TO CHO-AT<sub>1a</sub> CELLS

FIG. 2

COMPETITION OF COLD 12(S)-HETE FOR  
12(S)-[<sup>3</sup>H]HETE BINDING TO CHO-AT<sub>1a</sub> CELLS

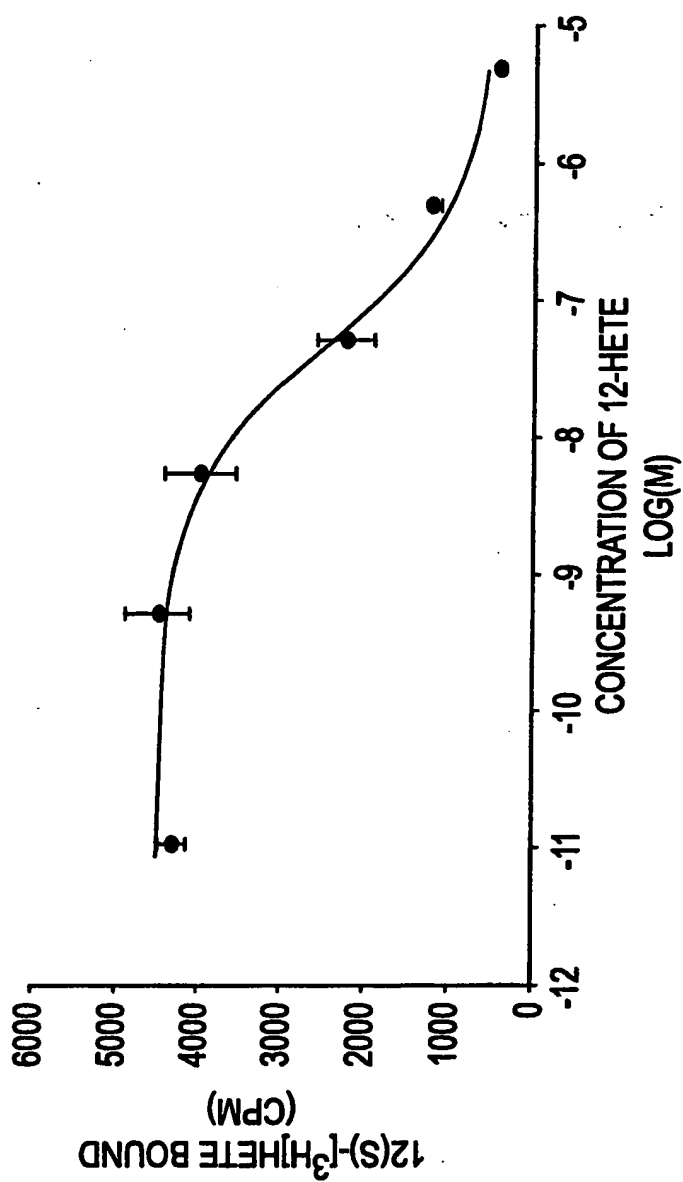
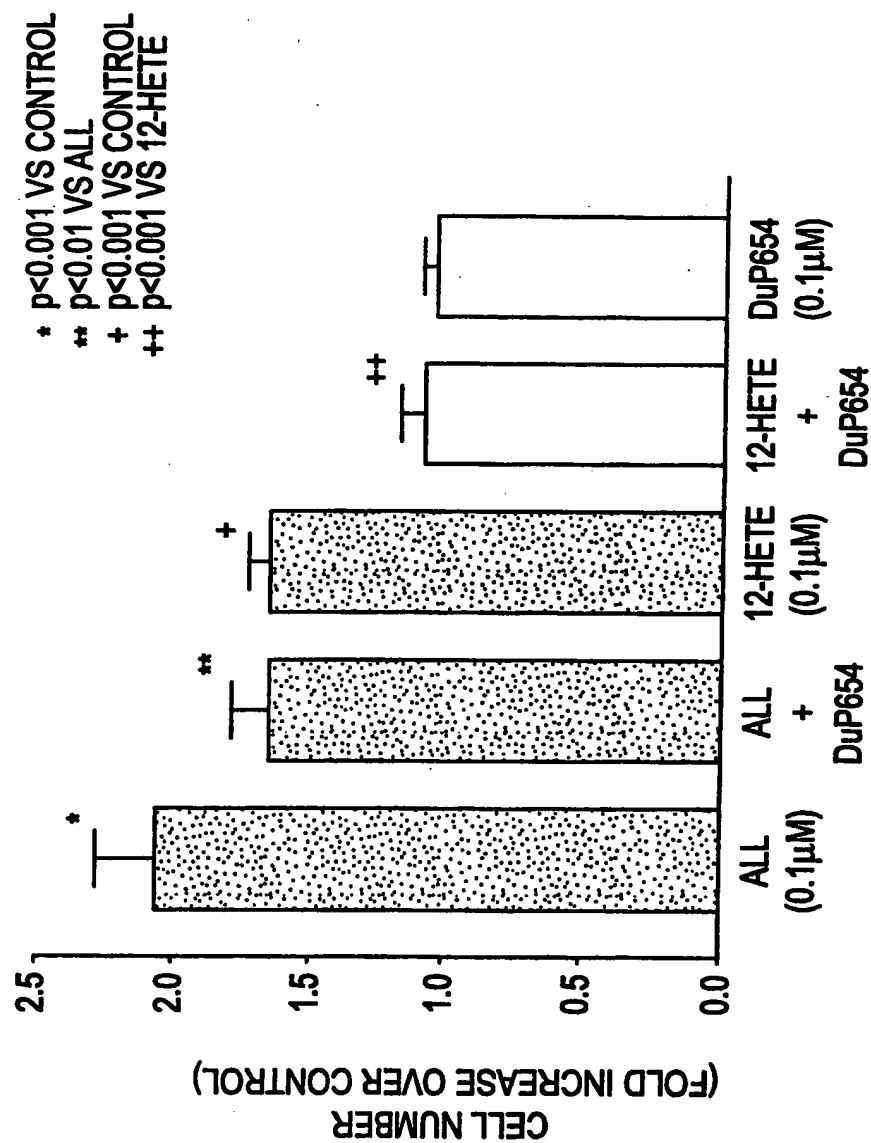


FIG. 3

EFFECT OF DuP654(12-HETE RECEPTOR ANTAGONIST) ON ALL  
AND 12-HETE-INDUCED GROWTH IN CHO-AT<sub>1a</sub> CELLS



**FIG. 4**  
EFFECT OF AGENTS ON 12(S)-[<sup>3</sup>H] HETE  
BINDING TO CHO-AT<sub>1a</sub> CELLS

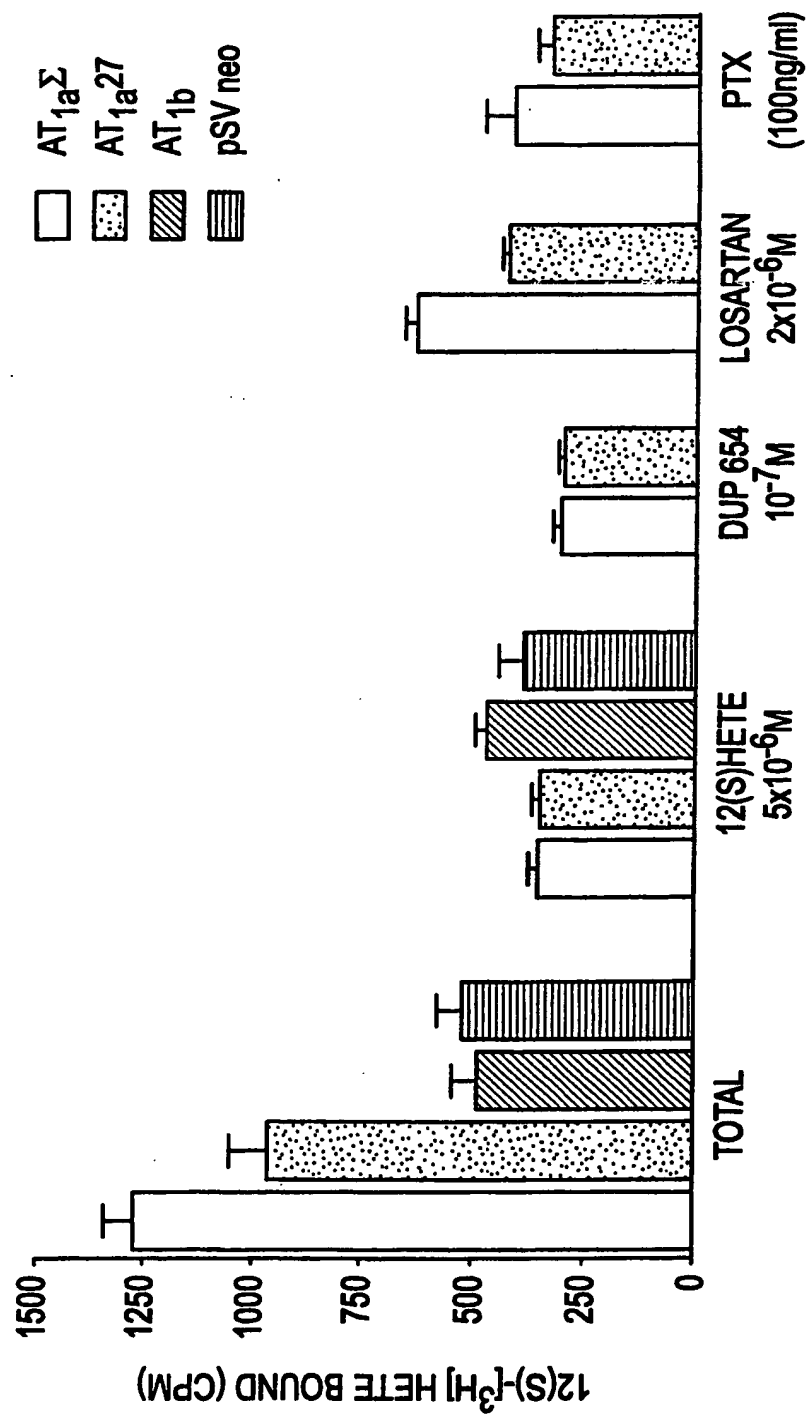
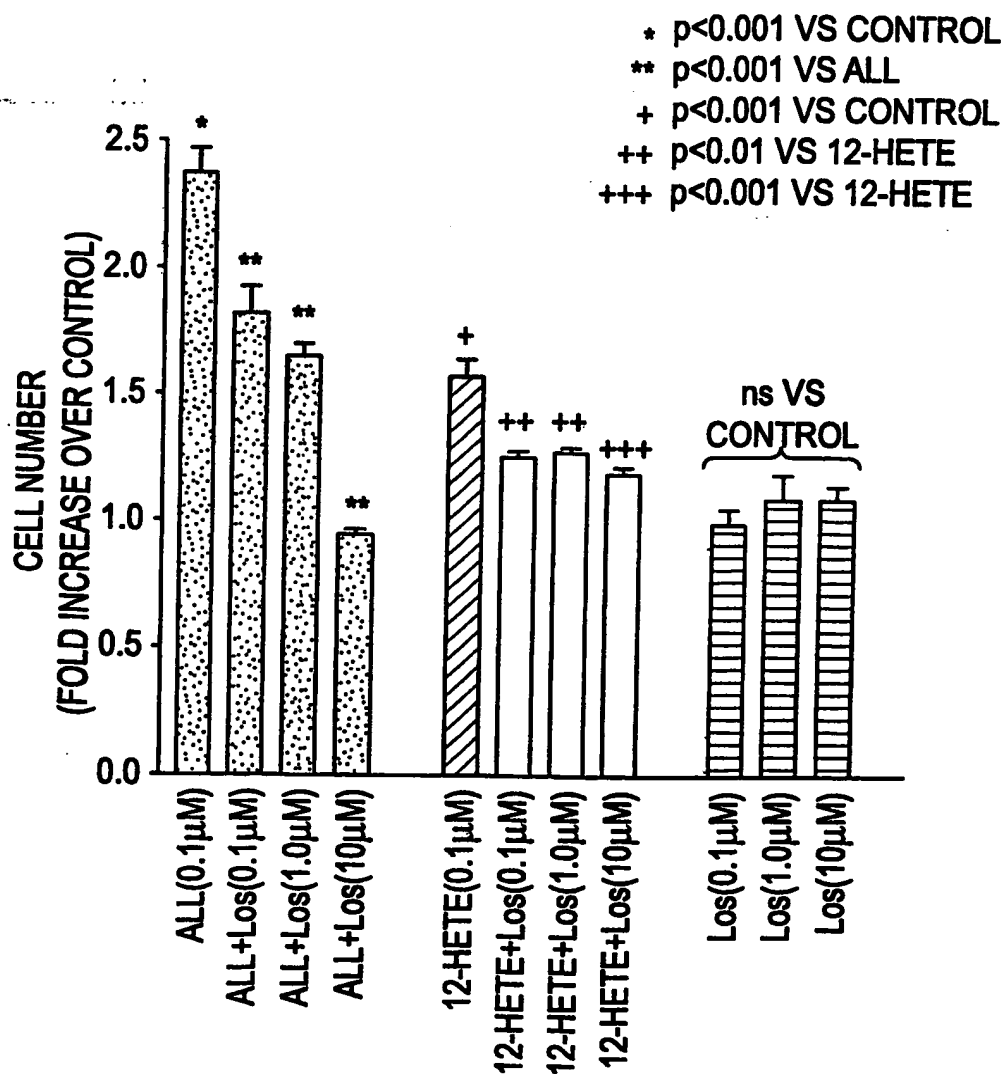


FIG. 5

EFFECT OF LOSARTAN(Los) ON ALL AND 12-HETE-  
INDUCED MITOGENESIS IN CHO-AT<sub>1a</sub> CELLS



SUBSTITUTE SHEET (RULE 26)

FIG. 6

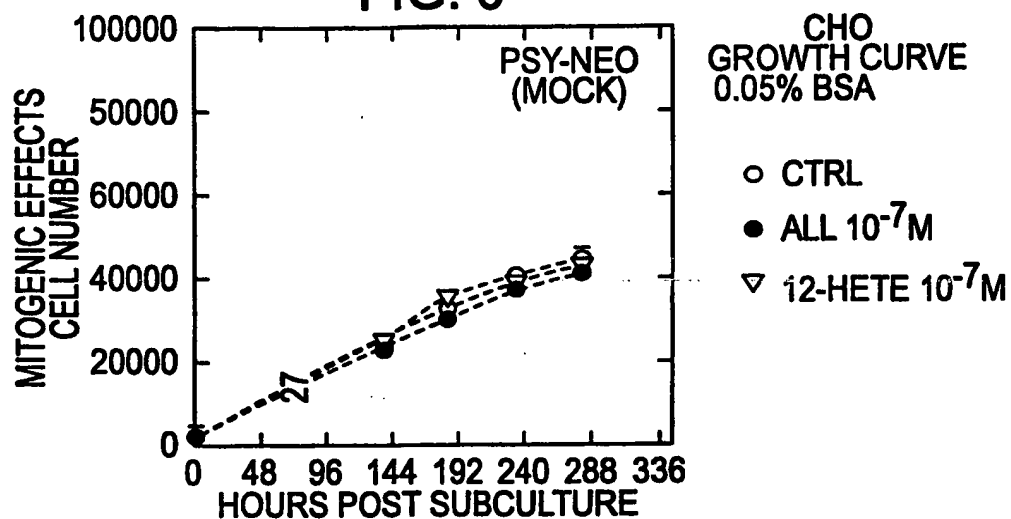


FIG. 7

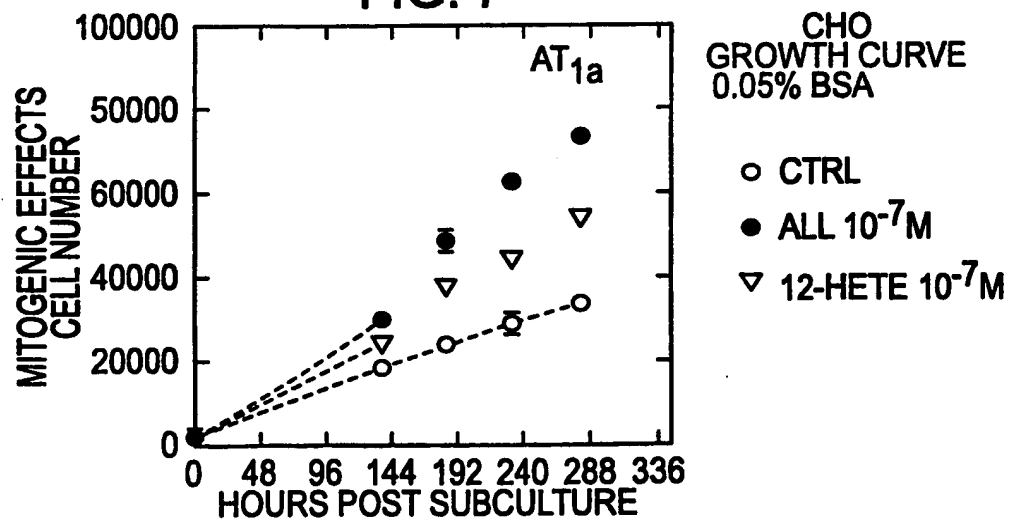




FIG. 8

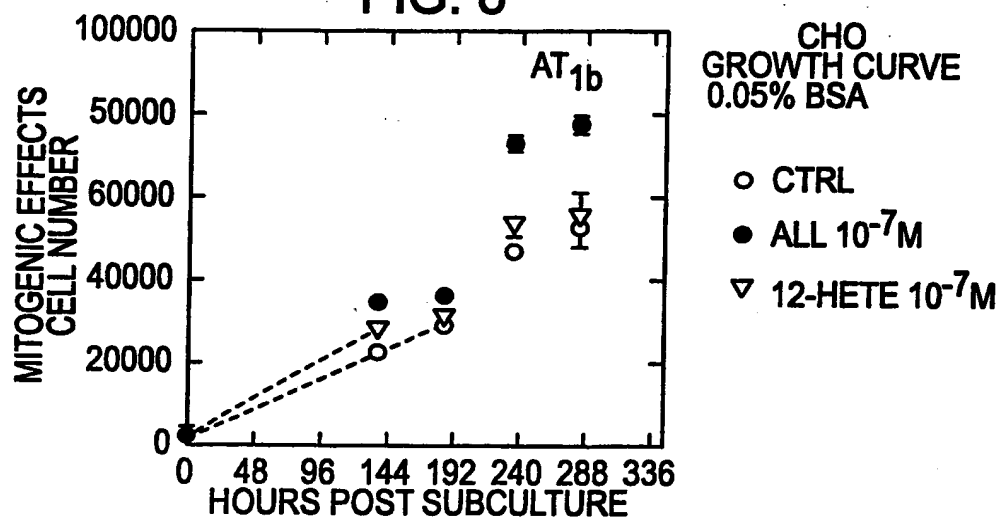


FIG. 9

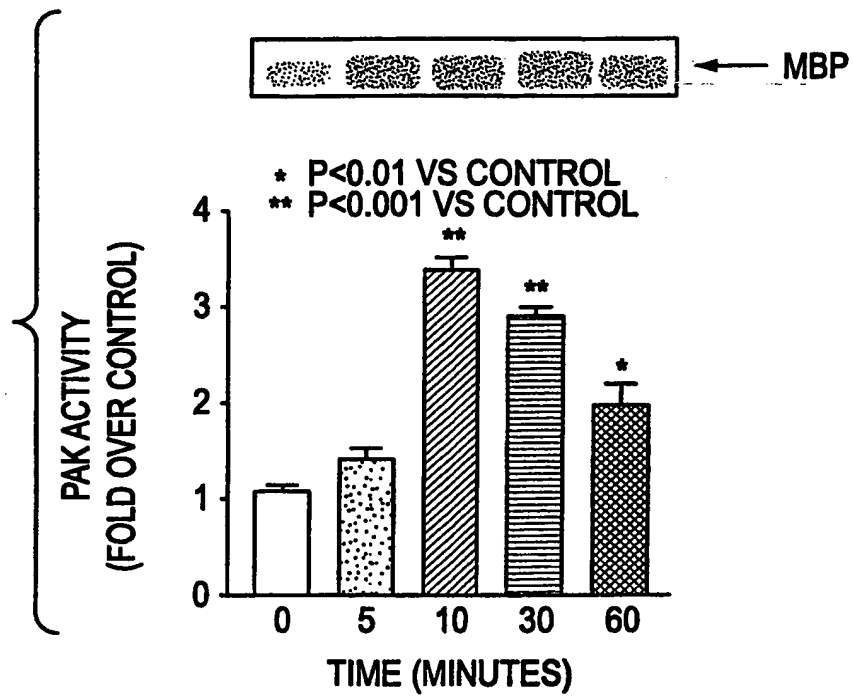


FIG. 10

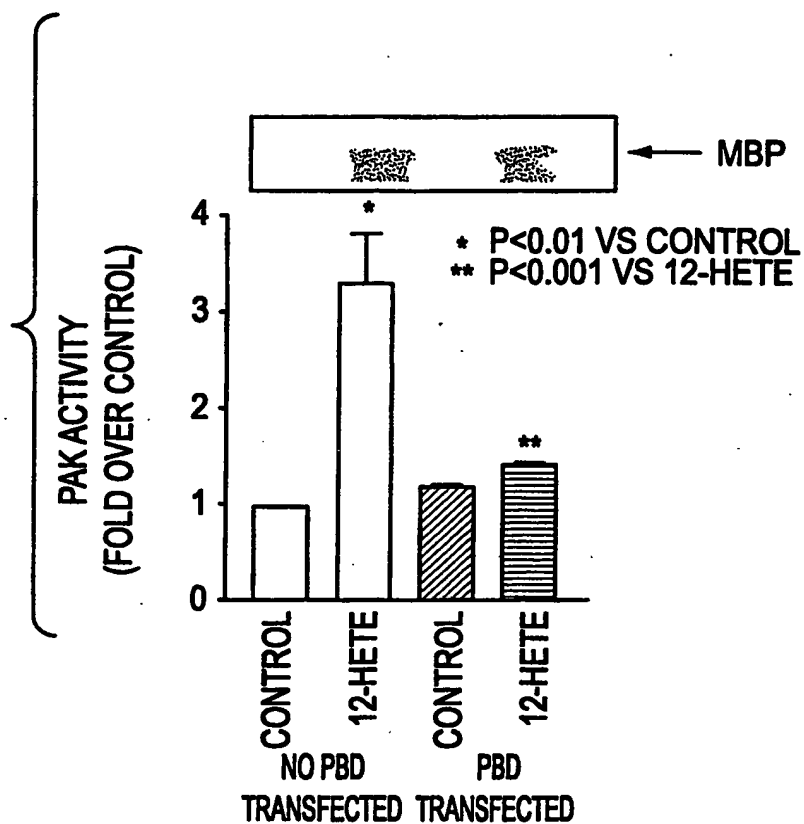
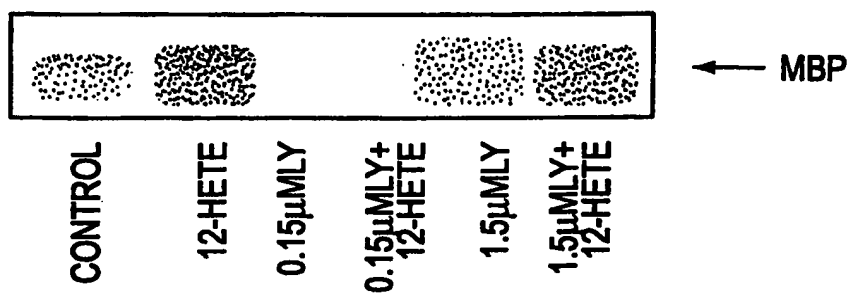


FIG. 11



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/21570

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K31/415 A61K38/16 A61K31/20 A61K39/395 A61K31/05

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 96 34943 A (HOPE CITY ;NADLER JERRY L (US); NATARAJAN RAMA (US)) 7 November 1996  see page 3, line 28 - page 5, line 29  see page 23, line 9 - page 24, line 29  see page 26, line 32 - page 27, line 27  see page 31, line 24 - line 27  see page 32, line 10 - line 16  see page 43, line 20 - line 22  see page 49, line 33 - line 34  see claims 6,21,25,29-31</p> <p style="text-align: center;">--- -/-</p>	1-22

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

17 February 1999

Date of mailing of the international search report

02/03/1999

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Authorized officer

Stein, A

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/21570

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARENBERGER, PETR ET AL: "The lipoxygenase inhibitor 2-phenylmethyl-1-naphthol (DuP 654) is a 12(S)-hydroxyeicosatetraenoic acid receptor antagonist in the human epidermal cell line SCL-II" SKIN PHARMACOL. (1993), 6(2), 148-51 , XP002093788 cited in the application see the whole document	1,2, 4-10,15, 17-22
Y		3,11
Y	BLEICH D ET AL: "The stress-activated c-jun protein kinase (JNK) is stimulated by lipoxygenase pathway product 12-HETE in RIN m5F cells" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATION, vol. 230, 1997, pages 448-451, XP002093789 cited in the application see the whole document	3,11
X	WEN Y ET AL: "Mechanisms of ANGII-induced mitogenic responses: role of 12-lipoxygenase and biphasic MAP kinase" AM.J.PHYSIOLOGY:CELL PHYSIOLOGY, vol. 40, no. 4, October 1996, pages c1212-c1220, XP002093790 cited in the application see the whole document	1-9,14, 18-22
A	NATARAJAN R ET AL: "MECHANISM OF ANGIOTENSIN II-INDUCED PROLIFERATION IN BOVINE ADRENOCORTICAL CELLS" ENDOCRINOLOGY, vol. 131, no. 3, September 1992, pages 1174-1180, XP000601236 see the whole document	1-15,21, 22

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/21570

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 1-22  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/21570

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9634943 A	07-11-1996	AU 5637796 A	21-11-1996
		CA 2220156 A	07-11-1996
		EP 0824583 A	25-02-1998
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Form PCT/ISA/210 (patent family annex) (July 1992)

